

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/30394 A1

- (51) International Patent Classification⁷: **A61K 48/00**, A61P 35/00, C12N 15/11, C07H 21/04
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- (21) International Application Number: PCT/AU00/01315
- (22) International Filing Date: 26 October 2000 (26.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
PQ 3676 26 October 1999 (26.10.1999) AU
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— With international search report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/30394 A1

(54) Title: TREATMENT OF CANCER

(57) Abstract: The present invention relates to a method for the treatment of tumours, the method comprising inhibiting angiogenesis in a subject in need thereof characterised in that angiogenesis is inhibited by administering to the subject an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR. The present invention also relates to a method of screening for agents which inhibits angiogenesis.

Treatment of cancer

FIELD OF THE INVENTION

5 The present invention relates to compositions and methods for the treatment of cancer.

BACKGROUND OF THE INVENTION

10 Cancer

Cancer accounted for over half a million deaths in the United States in 1998 alone, or approximately 23 % of all deaths (Landis et al., 1998). Only cardiovascular disease consistently claims more lives (Cotran et al., 1999).

15 There is growing evidence that the cellular and molecular mechanisms underlying tumour growth involves more than just tumour cell proliferation and migration. Importantly, tumour growth and metastasis are critically dependent upon ongoing angiogenesis, the process of new blood vessel formation (Crystal, 1999). Angiogenesis (also known as neovascularisation) is mediated by the migration and proliferation of vascular endothelial cells
20 that sprout from existing blood vessels to form a growing network of microvessels that supply growing tumours with vital nutrients. Primary solid tumours cannot grow beyond 1-2 mm diameter without active angiogenesis (Harris, 1998).

25 Human HepG2 hepatocellular carcinoma cells have been used as a model cancer cell line for the assessment of anti-neoplastic drugs (Yang et al., 1997). These cells basally and inducibly express the immediately-early gene and transcriptional regulator, early growth response factor-1 (EGR-1) (Kosaki et al., 1995).

30 Early Growth Response Protein (EGR-1)

Early growth response factor-1 (EGR-1, also known as Egr-1, NGFI-A, zif268, krox24 and TIS8) is the product of an immediate early gene and a prototypical member of the zinc finger family of transcriptional regulators (Gashler et al., 1995). Egr-1 binds to the promoters of a spectrum of genes
35 implicated in the pathogenesis of atherosclerosis and restenosis. These

include the platelet-derived growth factor (PDGF) A-chain (Khachigian et al., 1995), PDGF-B (Khachigian et al., 1996), transforming growth factor- β_1 (Liu et al., 1996, 1998), fibroblast growth factor-2 (FGF-2) (Hu et al., 1994; Biesiada et al., 1996), membrane type 1 matrix metalloproteinase (Haas et al., 1999),
5 tissue factor (Cui et al., 1996) and intercellular adhesion molecule-1 (Malzman et al., 1996). EGR-1 has also been localised to endothelial cells and smooth muscle cells in human atherosclerotic plaques (McCaffrey et al., 2000). Suppression of Egr-1 gene induction using sequence-specific catalytic DNA inhibits intimal thickening in the rat carotid artery following balloon
10 angioplasty (Santiago et al., 1999a).

DNAzymes

In human gene therapy, antisense nucleic acid technology has been one of the major tools of choice to inactivate genes whose expression causes
15 disease and is thus undesirable. The anti-sense approach employs a nucleic acid molecule that is complementary to, and thereby hybridizes with, an mRNA molecule encoding an undesirable gene. Such hybridization leads to the inhibition of gene expression.

Anti-sense technology suffers from certain drawbacks. Anti-sense
20 hybridization results in the formation of a DNA/target mRNA heteroduplex. This heteroduplex serves as a substrate for RNase H-mediated degradation of the target mRNA component. Here, the DNA anti-sense molecule serves in a passive manner, in that it merely facilitates the required cleavage by endogenous RNase H enzyme. This dependence on RNase H confers
25 limitations on the design of anti-sense molecules regarding their chemistry and ability to form stable heteroduplexes with their target mRNA's. Anti-sense DNA molecules also suffer from problems associated with non-specific activity and, at higher concentrations, even toxicity. An example of an alternative mechanism of antisense inhibition of target mRNA expression is
30 steric inhibition of movement of the translational apparatus along the mRNA.

As an alternative to anti-sense molecules, catalytic nucleic acid molecules have shown promise as therapeutic agents for suppressing gene expression, and are widely discussed in the literature (Haseloff (1988); Breaker (1994); Koizumi (1989); Otsuka; Kashani-Sabet (1992); Raillard
35 (1996); and Carmi (1996)). Thus, unlike a conventional anti-sense molecule, a catalytic nucleic acid molecule functions by actually cleaving its target

mRNA molecule instead of merely binding to it. Catalytic nucleic acid molecules can only cleave a target nucleic acid sequence if that target sequence meets certain minimum requirements. The target sequence must be complementary to the hybridizing arms of the catalytic nucleic acid, and the target must contain a specific sequence at the site of cleavage.

Catalytic RNA molecules ("ribozymes") are well documented (Haseloff (1988); Symonds (1992); and Sun (1997)), and have been shown to be capable of cleaving both RNA (Haseloff (1988)) and DNA (Raillard (1996)) molecules. Indeed, the development of in vitro selection and evolution techniques has made it possible to obtain novel ribozymes against a known substrate, using either random variants of a known ribozyme or random-sequence RNA as a starting point (Pan (1992); Tsang (1994); and Breaker (1994)).

Ribozymes, however, are highly susceptible to enzymatic hydrolysis within the cells where they are intended to perform their function. This in turn limits their pharmaceutical applications.

Recently, a new class of catalytic molecules called "DNAzymes" was created (Breaker and Joyce (1995); Santoro (1997)). DNAzymes are single-stranded, and cleave both RNA (Breaker (1994); Santoro (1997)) and DNA (Carmi (1996)). A general model for the DNAzyme has been proposed, and is known as the "10-23" model. DNAzymes following the "10-23" model, also referred to simply as "10-23 DNAzymes", have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. In vitro analyses show that this type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions under physiological conditions (Santoro (1997)).

DNAzymes show promise as therapeutic agents. However, DNAzyme success against a disease caused by the presence of a known mRNA molecule is not predictable. This unpredictability is due, in part, to two factors. First, certain mRNA secondary structures can impede a DNAzyme's ability to bind to and cleave its target mRNA. Second, the uptake of a DNAzyme by cells expressing the target mRNA may not be efficient enough to permit therapeutically meaningful results.

SUMMARY OF THE INVENTION

The present inventors have established that EGR-1 is critical in vascular endothelial cell replication and migration and that DNA-based, sequence-specific catalytic molecules targeting EGR-1 inhibit the growth of malignant cells in culture. These findings show that inhibitors of EGR or related EGR family members are useful in the treatment of tumours and that two separate mechanisms of action may be involved. Specifically, inhibitors of EGR family members may inhibit tumour growth indirectly by inhibiting angiogenesis and/or directly by blocking the EGR family member in tumour cells.

When used herein the term "EGR" refers to a member of the EGR family. Members of the EGR family are described in Gashler et al., 1995 and include EGR-1 to EGR-4. It is currently preferred that the EGR family member is EGR-1.

Accordingly, in a first aspect the present invention provides a method for the treatment of a tumour, the method comprising administering to a subject in need thereof an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.

In a second aspect, the present invention provides a method for inhibiting the growth or proliferation of a tumour cell, the method comprising contacting a tumour cell with an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.

In a third aspect, the present invention provides a tumour cell which has been transformed by introducing into the cell a nucleic acid molecule, the nucleic acid molecule comprising or encoding (i) an agent which inhibits induction of EGR, (ii) an agent which decreases expression of EGR, or (iii) an agent which decreases the nuclear accumulation or activity of EGR.

In a fourth aspect, the present invention provides a method of screening for an agent which inhibits angiogenesis, the method comprising testing a putative agent for the ability to inhibit induction of EGR, decrease expression of EGR or decrease the nuclear accumulation or activity of EGR.

In a preferred embodiment of the present invention the agent is selected from the group consisting of an EGR antisense oligonucleotide, a ribozyme targeted against EGR, a ssDNA targeted against EGR dsDNA such that the ssDNA forms a triplex with the EGR-1 ds DNA, and a DNAzyme targeted against EGR.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Insulin stimulates Egr-1-dependent gene expression in vascular endothelial cells. Growth-arrested bovine aortic endothelial cells previously
5 transfected with pEBS1³foscatt using FuGENE6 were incubated with D-glucose (5-30 mM), insulin (100 nM) or FGF-2 (25 ng/ml) as indicated for 24 h prior to preparation of cell lysates. CAT activity was normalized to the concentration of protein in the lysates.

Figure 2. Insulin-induced DNA synthesis in aortic endothelial cells is blocked
10 by antisense oligonucleotides targeting Egr-1. A, Insulin stimulates DNA synthesis. Growth-arrested endothelial cells were incubated with insulin (100 nM or 500 nM) or FBS (2.5%) for 18 h prior to ³H-thymidine pulse for a further 6 h. B, Antisense Egr-1 oligonucleotides inhibit insulin-inducible DNA synthesis.
15 Endothelial cells were incubated with 0.8 μ M of either AS2, AS2C or E3 prior to exposure to insulin (500 nM or 1000 nM) for 18 h and ³H-thymidine pulse for 6 h. C, Dose-dependent inhibition of insulin-inducible DNA synthesis. DNA synthesis stimulated by insulin (500 nM) was assessed in endothelial cells incubated with 0.4 μ M or 0.8 μ M of AS2 or AS2C. TCA-precipitable ³H-
20 thymidine incorporation into DNA was assessed using a β -scintillation counter.

Figure 3. Insulin-inducible DNA synthesis in cultured aortic endothelial cells
is MEK/ERK-dependent. Growth quiescent endothelial cells were preincubated
25 for 2 h with either PD98059 (10 μ M or 30 μ M), SB202190 (100 nM or 500 nM) or wortmannin (300 nM or 1000 nM) prior to the addition of insulin (500 nM) for 18 h and ³H-thymidine pulse. TCA-precipitable ³H-thymidine incorporation into DNA was assessed using a β -scintillation counter.

Figure 4. Wound repair after endothelial injury is potentiated by insulin in an
30 Egr-1-dependent manner. The population of cells in the denuded zone 3 d after injury in the various groups was quantitated and presented histodiagrammatically.

Figure 5. Human microvascular endothelial cell proliferation is inhibited by
35 DNA enzymes targeting human EGR-1. SV40-transformed HMEC-1 cells were grown in MCDB 131 medium with EGF (10 ng/ml) and hydrocortisone (1 μ g/ml)

supplements and 10% FBS. Forty-eight hours after incubation in serum-free medium without supplements, the cells were transfected with the indicated DNA enzyme (0.4 μ M) and transfected again 72 h after the change of medium, when 10% serum was added. The cells were quantitated by Coulter counter, 24 h after the addition of serum.

Figure 6. Sequence of NGFI-A DNAzyme (ED5), its scrambled control (ED5SCR) and 23 nt synthetic rat substrate. The translational start site is underlined.

Figure 7. NGFI-A DNAzyme inhibits the induction of NGFI-A protein by serum (FBS). Western blot analysis was performed using antibodies to NGFI-A, Sp1 or c-Fos. The Coomassie Blue stained gel demonstrates that uniform amounts of protein were loaded per lane. The sequence of EDC is 5'-CGC CAT TAG GCT AGC TAC AAC GAC CTA GTG AT-3' (SEQ ID NO:1); 3' T is inverted. SFM denotes serum-free medium.

Figure 8. SMC proliferation is inhibited by NGFI-A DNAzyme. **a**, Assessment of total cell numbers by Coulter counter. Growth-arrested SMCs that had been exposed to serum and/or DNAzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of AS2 is 5'-CTT GGC CGC TGC CAT-3' (SEQ ID NO:2). **b**, Proportion of cells incorporating Trypan Blue after exposure to serum and/or DNAzyme. Cells were stained incubated in 0.2% (w:v) Trypan Blue at 22 °C for 5 min prior to quantitation by hemocytometer in a blind manner. **c**, Effect of ED5 on pup SMC proliferation. Growth-arrested WKY12-22 cells exposed to serum and/or DNAzyme for 3 days were resuspended and numbers were quantitated by Coulter counter. Data is representative of 2 independent experiments performed in triplicate. The mean and standard errors of the mean are indicated in the figure. * indicates $P < 0.05$ (Student's paired t-test) as compared to control (FBS alone).

Figure 9. NGFI-A DNAzyme inhibition of neointima formation in the rat carotid artery. A neointima was achieved 18 days after permanent ligation of the right common carotid artery. DNAzyme (500 μ g) or vehicle alone was applied adventitiously at the time of ligation and again after 3 days. Sequence-specific inhibition of neointima formation. Neointimal and medial areas of 5 consecutive

sections per rat (5 rats per group) taken at 250 μ m intervals from the point of ligation were determined digitally and expressed as a ratio per group. The mean and standard errors of the mean are indicated by the ordinate axis. * denotes $P < 0.05$ as compared to the Lig, Lig+Veh or Lig+Veh+ED5SCR groups using the Wilcoxon rank sum test for unpaired data. Lig denotes ligation, Veh denotes vehicle.

Figure 10. HepG2 cell proliferation is inhibited by 0.75 μ M of DNAzyme DzA. Assessment of total cell numbers by Coulter counter. Growth-arrested cells that had been exposed to serum and/or DNAzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of DzA is 5'-caggggacaGGCTAGCTACAACGAcgttgcg (SEQ ID NO:3).

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the present invention provides a method for the treatment of a tumour, the method comprising administering to a subject in need thereof an agent which inhibits induction of an EGR, an agent which decreases
5 expression of an EGR or an agent which decreases the nuclear accumulation or activity of an EGR.

The method of the first aspect may involve indirect inhibition of tumour growth by inhibiting angiogenesis and/or direct inhibition by blocking EGR in
tumour cells.

10 In a preferred embodiment of the first aspect, the tumour is a solid tumour. The tumour may be selected from, without being limited to, a prostate tumour, a hepatocellular carcinoma, a skin carcinoma or a breast tumour.

As will be recognised by those skilled in this field there are a number
15 means by which the method of the present invention may be achieved.

In a preferred embodiment of the present invention, the EGR is EGR-1.

In one embodiment, the method is achieved by targeting the EGR gene directly using triple helix (triplex) methods in which a ssDNA molecule can bind to the dsDNA and prevent transcription.

20 In another embodiment, the method is achieved by inhibiting transcription of the EGR gene using nucleic acid transcriptional decoys. Linear sequences can be designed that form a partial intramolecular duplex which encodes a binding site for a defined transcriptional factor. Evidence suggests that EGR transcription is dependent upon the binding of Sp1, AP1 or
25 serum response factors to the promoter region. It is envisaged that inhibition of this binding of one or more of these transcription factors would inhibit transcription of the EGR gene.

In another embodiment, the method is achieved by inhibiting translation of the EGR mRNA using synthetic antisense DNA molecules that
30 do not act as a substrate for RNase H and act by sterically blocking gene expression.

In another embodiment, the method is achieved by inhibiting translation of the EGR mRNA by destabilising the mRNA using synthetic antisense DNA molecules that act by directing the RNase H-mediated
35 degradation of the EGR mRNA present in the heteroduplex formed between the antisense DNA and mRNA.

In one preferred embodiment of the present invention, the antisense oligonucleotide has a sequence selected from the group consisting of

- (i) ACA CTT TTG TCT GCT (SEQ ID NO:4), and
- (ii) CTT GGC CGC TGC CAT (SEQ ID NO:2).

5 In another embodiment, the method is achieved by inhibiting translation of the EGR mRNA by cleavage of the mRNA by sequence-specific hammerhead ribozymes and derivatives of the hammerhead ribozyme such as the Minizymes or Mini-ribozymes or where the ribozyme is derived from:

- (i) the hairpin ribozyme,
- 10 (ii) the Tetrahymena Group I intron,
- (iii) the Hepatitis Delta Viroid ribozyme or
- (iv) the Neurospora ribozyme.

It will be appreciated by those skilled in the art that the composition of the ribozyme may be;

- 15 (i) made entirely of RNA,
- (ii) made of RNA and DNA bases, or
- (iii) made of RNA or DNA and modified bases, sugars and backbones

Within the context of the present invention, the ribozyme may also be either;

- 20 (i) entirely synthetic or
- (ii) contained within a transcript from a gene delivered within a virus-derived vector, expression plasmid, a synthetic gene, homologously or heterologously integrated into the patients genome or delivered into cells ex vivo, prior to reintroduction of the cells of the patient, using
- 25 one of the above methods.

In another embodiment, the method is achieved by inhibition of the ability of the EGR gene to bind to its target DNA by expression of an antisense EGR-1 mRNA.

30 In another embodiment, the method is achieved by inhibition of EGR activity as a transcription factor using transcriptional decoy methods.

In another embodiment, the method is achieved by inhibition of the ability of the EGR gene to bind to its target DNA by drugs that have preference for GC rich sequences. Such drugs include nogalamycin, hedamycin and chromomycin A3 (Chiang et al J. Biol. Chem 1996;
35 271:23999).

In a preferred embodiment, the method is achieved by cleavage of EGR mRNA by a sequence-specific DNAzyme. In a further preferred embodiment, the DNAzyme comprises

- 5 (i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;
- (ii) a first binding domain contiguous with the 5' end of the catalytic domain; and
- (iii) a second binding domain contiguous with the 3' end of the catalytic domain,

10 wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:15, such that the DNAzyme cleaves the EGR mRNA.

As used herein, "DNAzyme" means a DNA molecule that specifically
15 recognizes and cleaves a distinct target nucleic acid sequence, which may be either DNA or RNA.

In a preferred embodiment, the binding domains of the DNAzyme are complementary to the regions immediately flanking the cleavage site. It will be appreciated by those skilled in the art, however, that strict
20 complementarity may not be required for the DNAzyme to bind to and cleave the EGR mRNA.

The binding domain lengths (also referred to herein as "arm lengths") can be of any permutation, and can be the same or different. In a preferred embodiment, the binding domain lengths are at least 6 nucleotides.
25 Preferably, both binding domains have a combined total length of at least 14 nucleotides. Various permutations in the length of the two binding domains, such as 7+7, 8+8 and 9+9, are envisioned.

The catalytic domain of a DNAzyme of the present invention may be any suitable catalytic domain. Examples of suitable catalytic domains are
30 described in *Santoro and Joyce, 1997* and U.S. Patent No. 5,807,718. In a preferred embodiment, the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA (SEQ ID NO:5).

Within the context of the present invention, preferred cleavage sites within the region of EGR mRNA corresponding to nucleotides 168 to 332 are
35 as follows:

- (i) the GU site corresponding to nucleotides 198-199;

- (ii) the GU site corresponding to nucleotides 200-201;
- (iii) the GU site corresponding to nucleotides 264-265;
- (iv) the AU site corresponding to nucleotides 271-272;
- (v) the AU site corresponding to nucleotides 292-293;
- 5 (vi) the AU site corresponding to nucleotides 301-302;
- (vii) the GU site corresponding to nucleotides 303-304; and
- (viii) the AU site corresponding to nucleotides 316-317.

In a further preferred embodiment, the DNAzyme has a sequence
10 selected from:

- (i) 5'-caggggacaGGCTAGCTACAACGAcgttgcg (SEQ ID NO:3)
targets GU (bp 198, 199); arms hybridise to bp 189-207
- (ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO:6)
15 targets GU (bp 200, 201); arms hybridise to bp 191-209
- (iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO:7)
targets GU (bp 264, 265); arms hybridise to bp 255-273
- 20 (iv) 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8)
targets AU (bp 271, 272); arms hybridise to bp 262-280
- (v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO:9)
targets AU (bp 271, 272); arms hybridise to bp 262-280
- 25 (vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO:10)
targets AU (bp 301, 302); arms hybridise to bp 292-310
- (vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO:11)
30 targets GU (bp 303, 304); arms hybridise to bp 294-312
- (viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO:12)
targets AU (bp 316, 317); arms hybridise to bp 307-325.

In a particularly preferred embodiment, the DNAzyme targets the the GU site corresponding to nucleotides 198-199, the AU site corresponding to nucleotides 271-272 or the AU site corresponding to nucleotides 301-302.

In a further preferred embodiment, the DNAzyme has the sequence:

- 5 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3),
5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO:10),
5'-ccgcgccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8) or
5'-ccgctgccaGGCTAGCTACAACGAcccgacgt (SEQ ID NO:9).

- 10 In applying DNAzyme-based treatments, it is preferable that the DNAzymes be as stable as possible against degradation in the intra-cellular milieu. One means of accomplishing this is by incorporating a 3'-3' inversion at one or more termini of the DNAzyme. More specifically, a 3'-3' inversion (also referred to herein simply as an "inversion") means the covalent phosphate bonding between the 3' carbons of the terminal nucleotide and its
15 adjacent nucleotide. This type of bonding is opposed to the normal phosphate bonding between the 3' and 5' carbons of adjacent nucleotides, hence the term "inversion". Accordingly, in a preferred embodiment, the 3'-end nucleotide residue is inverted in the building domain contiguous with the 3' end of the catalytic domain. In addition to inversions, the instant
20 DNAzymes may contain modified nucleotides. Modified nucleotides include, for example, N3'-P5' phosphoramidate linkages, and peptide-nucleic acid linkages. These are well known in the art.

In a particularly preferred embodiment, the DNAzyme includes an inverted T at the 3' position.

- 25 Although the subject may be any animal or human, it is preferred that the subject is a human.

- Within the context of the present invention, the EGR inhibitory agents may be administered either alone or in combination with one or more additional anti-cancer agents which will be known to a person skilled in the
30 art.

- Administration of the inhibitory agents may be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be performed, for example, intravenously, orally, via implant, transmucosally, transdermally, topically,
35 intramuscularly, subcutaneously or extracorporeally. In addition, the instant pharmaceutical compositions ideally contain one or more routinely used

pharmaceutically acceptable carriers. Such carriers are well known to those skilled in the art. The following delivery systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition. In one embodiment
5 the delivery vehicle contains Mg^{2+} or other cation(s) to serve as co-factor(s) for efficient DNAzyme bioactivity.

Transdermal delivery systems include patches, gels, tapes and creams, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), hydrophilic
10 polymers (e.g., polycarbophil and polyvinylpyrrolidone), and adhesives and tackifiers (e.g., polyisobutylenes, silicone-based adhesives, acrylates and polybutene).

Transmucosal delivery systems include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers
15 and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Oral delivery systems include tablets and capsules. These can contain
20 excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinylpyrrolidone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

Solutions, suspensions and powders for reconstitutable delivery
25 systems include vehicles such as suspending agents (e.g., gums, xanthans, cellulose and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine), preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, coating
30 agents, and chelating agents (e.g., EDTA).

Topical delivery systems include, for example, gels and solutions, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic
35 polymers (e.g., polycarbophil and polyvinylpyrrolidone). In the preferred embodiment, the pharmaceutically acceptable carrier is a liposome or a

biodegradable polymer. Examples of carriers which can be used in this invention include the following: (1) Eugene6[®] (Roche); (2) SUPERFECT[®](Qiagen); (3) Lipofectamine 2000[®](GIBCO BRL); (4) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N,N,N-tetramethyl-N,N,N,N-tetrapalmitylspermine and dioleoyl phosphatidyl-ethanolamine (DOPE)(GIBCO BRL); (5) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (6) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethyl-ammoniummethylsulfate] (Boehringer Mannheim); and (7) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

In a preferred embodiment, the agent is injected into or proximal the solid tumour. Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g., ethanol, propylene glycol and sucrose) and polymers (e.g., polycaprylactones and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprylactone.

Delivery of the nucleic acid agents described may also be achieved via one or more, of the following non-limiting examples of vehicles:

- (a) liposomes and liposome-protein conjugates and mixtures;
- (b) non-liposomal lipid and cationic lipid formulations;
- (c) activated dendrimer formulations;
- (d) within polymer formulations such pluronic gels or within ethylene vinyl acetate copolymer (EVAc). The polymer may be delivered intra-luminally;
- (e) within a viral-liposome complex, such as Sendai virus; or
- (f) as a peptide-DNA conjugate.

Determining the prophylactically effective dose of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the prophylactically effective dose contains between about 0.1 mg and about 1 g of the instant DNAzyme. In another embodiment, the prophylactically effective dose contains between about 1 mg and about 100 mg of the instant DNAzyme. In a further embodiment, the prophylactically effective dose contains between about 10 mg and about 50 mg of the instant DNAzyme. In yet a further embodiment,

the prophylactically effective does contains about 25 mg of the instant DNAzyme.

It is also envisaged that nucleic acid agents targeting EGR may be administered by *ex vivo* transfection of cell suspensions, thereby inhibiting
5 tumour growth, differentiation and/or metastasis.

In a second aspect, the present invention provides a method for inhibiting the growth or proliferation of a tumour cell, the method comprising contacting a tumour cell with an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which
10 decreases the nuclear accumulation or activity of EGR.

In a third aspect, the present invention provides a tumour cell which has been transformed by introducing into the cell a nucleic acid molecule, the nucleic acid molecule comprising or encoding (i) an agent which inhibits induction of EGR, (ii) an agent which decreases expression of EGR, or (iii) an
15 agent which decreases the nuclear accumulation or activity of EGR.

In a preferred embodiment of the third and fourth aspects, the agent is selected from the group consisting of an EGR antisense oligonucleotide or mRNA, a sequence-specific ribozyme targeted against EGR, a ssDNA targeted against EGR dsDNA and a sequence specific DNAzyme targeted against EGR.
20

In a fourth aspect, the present invention provides a method of screening for an agent which inhibits angiogenesis, the method comprising testing a putative agent for the ability to inhibit induction of EGR, decrease expression of EGR or decrease the nuclear accumulation or activity of EGR.

The putative agent may be tested for the ability to inhibit EGR by any
25 suitable means. For example, the test may involve contacting a cell which expresses EGR with the putative agent and monitoring the production of EGR mRNA (by, for example, Northern blot analysis) or EGR protein (by, for example, immunohistochemical analysis or Western blot analysis). Other suitable tests will be known to those skilled in the art.

For reference, Table 1 below sets forth a comparison between the DNA sequences of mouse, rat and human EGR-1.

Table 1
Mouse, Rat and Human EGR-1

5

Mouse, Rat and Human EGR-1

Symbol comparison table: GenRunData:pileupdna.cmp CompCheck: 6876

GapWeight: 5.000

10 GapLengthWeight: 0.300

EGR1align.msf MSF: 4388 Type: N April 7, 1998 12:07 Check: 5107

Name: mouseEGR1 Len: 4388 Check: 8340 Weight: 1.00 (SEQ ID NO:13)

Name: ratEGR1 Len: 4388 Check: 8587 Weight: 1.00 (SEQ ID NO:14)

15 Name: humanEGR1 Len: 4388 Check: 8180 Weight: 1.00 (SEQ ID NO:15)

NB. THIS IS RAT NGFI-A numbering

1 50

mouseEgr1

20 ratNGFIA CCGCGGAGCC TCAGCTCTAC GCGCCTGGCG CCCTCCCTAC GCGGGCGTCC

humanEGR1

51 100

mouseEGR1

25 ratEGR1 CCGACTCCCG CGCGCGTTCA GGCTCCGGGT TGGGAACCAA GGAGGGGGAG

humanEGR1

101 150

30 mouseEGR1

ratEGR1 GGTGGGTGCG CCGACCCGGA AACACCATAT AAGGAGCAGG AAGGATCCCC

humanEGR1

151 200

35 mouseEGR1

ratEGR1 CGCCGGAACA GACCTTATTT GGGCAGCGCC TTATATGGAG TGGCCCAATA

humanEGR1

201 250

40 mouseEGR1

ratEGR1 TGGCCCTGCC GCTTCCGGCT CTGGGAGGAG GGGCGAACGG GGGTTGGGGC

humanEGR1

251 300

45 mouseEGR1

ratEGR1 GGGGGCAAGC TGGGAACTCC AGGAGCCTAG CCCGGGAGGC CACTGCCGCT

humanEGR1

301 350

50 mouseEGR1

ratEGR1 GTTCCAATAC TAGGCTTTCC AGGAGCCTGA GCGCTCAGGG TGCCGGAGCC

humanEGR1

351 400

55 mouseEGR1

ratEGR1 GGTCGCAGGG TGGAAGCGCC CACCGCTCTT GGATGGGAGG TCTTCACGTC

humanEGR1

401 450

mouseEGR1

	rateEGR1	ACTCCGGGTC	CTCCCGGTCG	GTCCTTCCAT	ATTAGGGCTT	CCTGCTTCCC
	humanEGR1
		451				500
5	mouseEGR1
	rateEGR1	ATATATGGCC	ATGTACGTCA	CGGCGGAGGC	GGGCCCGTGC	TGTTTCAGAC
	humanEGR1
		501				550
10	mouseEGR1
	rateEGR1	CCTTGAAATA	GAGGCCGATT	CGGGGAGTCG	CGAGAGATCC	CAGCGCGCAG
	humanEGR1CCGCAG
		551				600
15	mouseEGR1GGGGA	GCCGCCGCCG	CGATTTCGCCG	CCGCCGCCAG	CTTCCGCCGC
	rateEGR1	AACTTGGGGA	GCCGCCGCCG	CGATTTCGCCG	CCGCCGCCAG	CTTCCGCCGC
	humanEGR1	AACTTGGGGA	GCCGCCGCCG	CCATCCGCCG	CCGCAGCCAG	CTTCCGCCGC
		601				650
20	mouseEGR1	CGCAAGATCG	GCCCCTGCCC	CAGCCTCCGC	GGCAGCCCTG	CGTCCACCAC
	rateEGR1	CGCAAGATCG	GCCCCTGCCC	CAGCCTCCGC	GGCAGCCCTG	CGTCCACCAC
	humanEGR1	CGCAGGACCG	GCCCCTGCCC	CAGCCTCCGC	AGCCGCGGCG	CGTCCACGCC
		651				700
25	mouseEGR1	GGGCCGCGGC	TACCGCCAGC	CTGGGGGCCC	ACCTACACTC	CCCGCAGTGT
	rateEGR1	GGGCCGCGGC	CACCGCCAGC	CTGGGGGCCC	ACCTACACTC	CCCGCAGTGT
	humanEGR1	CGCCCGCGCC	CAGGGCGAGT	CGGGGTGCGC	GCCTGCACGC	TTCTCAGTGT
		701				750
30	mouseEGR1	GCCCCCTGCAC	CCCGCATGTA	ACCCGGCCAA	CCCCCGGCGA	GTGTGCCCTC
	rateEGR1	GCCCCCTGCAC	CCCGCATGTA	ACCCGGCCAA	CATCCGGCGA	GTGTGCCCTC
	humanEGR1	TCCCC.GCGC	CCCGCATGTA	ACCCGGCCAG	GCCCCCGCAA	CGGTGTCCCC
		751				800
35	mouseEGR1	AGTAGCTTCG	GCCCCGGGCT	GCGCCCACC.	.ACCCAACAT	CAGTTCCTCCA
	rateEGR1	AGTAGCTTCG	GCCCCGGGCT	GCGCCCACC.	.ACCCAACAT	CAGTTCCTCCA
	humanEGR1	TGCAGCTCCA	GCCCCGGGCT	GCACCCCCC	GCCCCGACAC	CAGTTCCTCCA
		801				850
40	mouseEGR1	GCTCGCTGGT	CCGGGATGGC	AGCGGCCAAG	GCCGAGATGC	AATTGATGTC
	rateEGR1	GCTCGCACGT	CCGGGATGGC	AGCGGCCAAG	GCCGAGATGC	AATTGATGTC
	humanEGR1	GCCTGCTCGT	CCAGGATGGC	CGCGGCCAAG	GCCGAGATGC	AGCTGATGTC
45	ED5 (rat) arms hybridise to bp 807-825 in rat sequ hED5(hum) arms hybridise to bp 262-280 in hum sequ					
		851				900
	mouseEGR1	TCCGCTGCAG	ATCTCTGACC	CGTTCGGCTC	CTTTCCTCAC	TCACCCACCA
	rateEGR1	TCCGCTGCAG	ATCTCTGACC	CGTTCGGCTC	CTTTCCTCAC	TCACCCACCA
50	humanEGR1	CCCGCTGCAG	ATCTCTGACC	CGTTCGGATC	CTTTCCTCAC	TCGCCACCA
		901				950
	mouseEGR1	TGGACAACTA	CCCCAAACTG	GAGGAGATGA	TGCTGCTGAG	CAACGGGGCT
	rateEGR1	TGGACAACTA	CCCCAAACTG	GAGGAGATGA	TGCTGCTGAG	CAACGGGGCT
55	humanEGR1	TGGACAACTA	CCCTAAGCTG	GAGGAGATGA	TGCTGCTGAG	CAACGGGGCT
		951				1000
	mouseEGR1	CCCCAGTTCC	TCGGTGCTGC	CGGAACCCCA	GAGGGCAGCG	GCGGTAAT..
	rateEGR1	CCCCAGTTCC	TCGGTGCTGC	CGGAACCCCA	GAGGGCAGCG	GCGGCAATAA
60	humanEGR1	CCCCAGTTCC	TCGGCGCCGC	CGGGGCCCA	GAGGGCAGCG	GCAGCAACAG

		1001				1050
	mouseEGR1AGC	AGCAGCAGCA	CCAGCAGCGG	GGGCGGTGGT	GGGGGCGGCA
	ratEGR1	CAGCAGCAGC	AGCAGCAGCA	GCAGCAGCGG	GGGCGGTGGT	GGGGGCGGCA
5	humanEGR1	CAGCAGCAGC	AGCAGCAGGG	GCGGTGGAGG	CGGCGGGGGC	GGCAGCAACA
		1051				1100
	mouseEGR1	GCAACAGCGG	CAGCAGCGCC	TTCAATCCTC	AAGGGGAGCC	GAGCGAACAA
	ratEGR1	GCAACAGCGG	CAGCAGCGCT	TTCAATCCTC	AAGGGGAGCC	GAGCGAACAA
10	humanEGR1	GCAGCAGCAG	CAGCAGCACC	TTCAACCCTC	AGGCGGACAC	GGGCGAGCAG
		1101				1150
	mouseEGR1	CCCTATGAGC	ACCTGACCAC	AG...AGTCC	TTTTCTGACA	TCGCTCTGAA
	ratEGR1	CCCTACGAGC	ACCTGACCAC	AGGTAAGCGG	TGGTCTGCGC	CGAGGCTGAA
15	humanEGR1	CCCTACGAGC	ACCTGACCGC	AG...AGTCT	TTTCCTGACA	TCTCTCTGAA
		1151				1200
	mouseEGR1	TAATGAGAAG	GCGATGGTGG	AGACGAGTTA	TCCCAGCCAA	ACGACTCGGT
	ratEGR1	TCCCCCTTCG	TGACTACCTT	AACGTCCAGT	CCTTTGCAGC	ACGGACCTGC
20	humanEGR1	CAACGAGAAG	GTGCTGGTGG	AGACCAGTTA	CCCCAGCCAA	ACCACTCGAC
		1201				1250
	mouseEGR1	TGCCTCCCAT	CACCTATACT	GGCCGCTTCT	CCCTGGAGCC	CGCACCCAAC
	ratEGR1	ATCTAGATCT	TAGGGACGGG	ATTGGGATTT	CCCTCTATTC	..CACACAGC
25	humanEGR1	TGCCCCCCAT	CACCTATACT	GGCCGCTTTT	CCCTGGAGCC	TGCACCCAAC
		1251				1300
	mouseEGR1	AGTGGCAACA	CTTTGTGGCC	TGAACCCCTT	TTCAGCCTAG	TCAGTGGCCT
	ratEGR1	TCCAGGGACT	TGTGTTAGAG	GGATGTCTGG	GGACCCCCCA	ACCTTCCATC
30	humanEGR1	AGTGGCAACA	CCTTGTGGCC	CGAGCCCCCT	TTCAGCTTGG	TCAGTGGCCT
		1301				1350
	mouseEGR1	CGTGAGCATG	ACCAATCCTC	CGACCTCTTC	ATCCTCGGCG	CCTTCTCCAG
	ratEGR1	CTTGCGGGTG	CGCGGAGGGC	AGACCGTTTG	TTTTGGATGG	AGAACTCAAG
35	humanEGR1	AGTGAGCATG	ACCAACCCAC	CGGCCTCCTC	GTCTCTAGCA	CCATCTCCAG
		1351				1400
	mouseEGR1	CTGCTTCATC	GTCTTCCTCT	GCCTCCCAGA	GCCCGCCCCCT	GAGCTGTGCC
	ratEGR1	TTGCGTGGGT	GGCT.....GGAGT	GGGGGAGGGT	TTGTTTTGAT
40	humanEGR1	CGGCCTCCTC	CGC...CTCC	GCCTCCCAGA	GCCCACCCCT	GAGCTGCGCA
		1401				1450
	mouseEGR1	GTGCCGTCCA	ACGACAGCAG	TCCCATCTAC	TCGGCTGCGC	CCACCTTTCC
	ratEGR1	GAGCAGGGTT	GC....CCCC	TCCCCCGCGC	GCGTTGTCGC	GAGCCTTGTT
45	humanEGR1	GTGCCATCCA	ACGACAGCAG	TCCCATTATC	TCAGCGGCAC	CCACCTTCCC
		1451				1500
	mouseEGR1	TACTCCCAAC	ACTGACATTT	TTCCTGAGCC	CCAAAGCCAG	GCCTTTCCTG
	ratEGR1	TGCAGCTTGT	TCCAAGGAA	GGGCTGAAAT	CTGTCACCAG	GGATGTCCCG
50	humanEGR1	CACGCCGAAC	ACTGACATTT	TCCCTGAGCC	ACAAAGCCAG	GCCTTCCCGG
		1501				1550
	mouseEGR1	GCTCGGCAGG	CACAGCCTTG	CAGTACCCGC	CTCCTGCCTA	CCCTGCCACC
	ratEGR1	CCGCCCAGGG	TAGGGGCGCG	CATTAGCTGT	GGCC..ACTAG	GGTGCTGGCG
55	humanEGR1	GCTCGGCAGG	GACAGCGCTC	CAGTACCCGC	CTCCTGCCTA	CCCTGCCGCC
		1551				1600
	mouseEGR1	AAAGGTGGTT	TCCAGGTTCC	CATGATCCCT	GACTATCTGT	TTCCACAACA
	ratEGR1	GGATTCCCTC	ACCCCGGACG	CCTGCTGCGG	AGCGCTCTCA	GAGCTGCAGT
60	humanEGR1	AAGGGTGGCT	TCCAGGTTCC	CATGATCCCC	GACTACCTGT	TTCCACAGCA

		1601				1650
	mouseEGR1	ACAGGGGAGAC	CTGAGCCTGG	GCACCCAGAG	CCAGAAGCCC	TTCCAGGGTC
	ratEGR1	AGAGGGGGGAT	TCTCTGTTTG	CGTCAGCTGT	CGAAATGGCT	CT.....GC
5	humanEGR1	GCAGGGGGAT	CTGGGCCTGG	GCACCCAGAG	CCAGAAGCCC	TTCCAGGGCC
		1651				1700
	mouseEGR1	TGGAGAACCG	TACCCAGCAG	CCTTCGCTCA	CTCCACTATC	CACTATTAAA
	ratEGR1	CACTGGAGCA	GGTCCAGGAA	CATTGCAATC	TGCTGCTATC	AATTATTAAC
10	humanEGR1	TGGAGAGCCG	CACCCAGCAG	CCTTCGCTAA	CCCCTCTGTC	TACTATTAAG
		1701				1750
	mouseEGR1	GCCTTCGCCA	CTCAGTCGGG	CTCCCAGGAC	TTAAAG....	...GCTCTTA
	ratEGR1	CACATCGAGA	GTCAGTGGTA	GCCGGGCGAC	CTCTTGCCCTG	GCCGCTTCGG
15	humanEGR1	GCCTTTGCCA	CTCAGTCGGG	CTCCCAGGAC	CTGAAG....	...GCCCTCA
		1751				1800
	mouseEGR1	ATACCACCTA	CCAATCCCAG	CTCATCA..A	ACCCAGCCGC	ATGCGCAAGT
	ratEGR1	CTCTCATCGT	CCAGTGATTG	CTCTCCAGTA	ACCAGGCCCTC	TCTGTTCTCT
20	humanEGR1	ATACCAGCTA	CCAGTCCCAG	CTCATCA..A	ACCCAGCCGC	ATGCGCAAGT
		1801				1850
	mouseEGR1	ACCCCAACCG	GCCCAGCAAG	ACACCCCCC	ATGAACGCCC	ATATGCTTGC
	ratEGR1	TTCTTGCCAG	AGTCCTTTTC	TGACATCGCT	CTGAATAACG	AGAAG..GCG
25	humanEGR1	ATCCCAACCG	GCCCAGCAAG	ACGCCCCCCC	ACGAACGCCC	TTACGCTTGC
		1851				1900
	mouseEGR1	CCTGTCGAGT	CCTGCGATCG	CCGCTTTTCT	CGCTCGGATG	AGCTTACCCG
	ratEGR1	CTGGTGGAGA	CAAGTTATCC	CAGCCAAACT	ACCCGGTTGC	CTCCCATCAC
30	humanEGR1	CCAGTGGAGT	CCTGTGATCG	CCGCTTCTCC	CGCTCCGACG	AGCTACCCG
		1901				1950
	mouseEGR1	CCATATCCGC	ATCCACACAG	GCCAGAAGCC	CTTCCAGTGT	CGAATCTGCA
	ratEGR1	CTATACTGGC	CGCTTCTCCC	TGGAGCCTGC	ACCCAACAGT	GGCAACACTT
35	humanEGR1	CCACATCCGC	ATCCACACAG	GCCAGAAGCC	CTTCCAGTGC	CGCATCTGCA
		1951				2000
	mouseEGR1	TGCGTAACTT	CAGTCGTAGT	GACCACCTTA	CCACCCACAT	CCGCACCCAC
	ratEGR1	TGTGGCCTGA	ACCCCTTTTC	AGCCTAGTCA	GTGGCCTTGT	GAGCATGACC
40	humanEGR1	TGCGCAACTT	CAGCCGCAGC	GACCACCTCA	CCACCCACAT	CCGCACCCAC
		2001				2050
	mouseEGR1	ACAGGCGAGA	AGCCTTTTGC	CTGTGACATT	TGTGGGAGGA	AGTTTGCCAG
	ratEGR1	AACCCCTCAA	CCTCTTCATC	CTCAGCGCCT	TCTCCAGCTG	CTTCATCGTC
45	humanEGR1	ACAGGCGAAA	AGCCCTTCGC	CTGCGACATC	TGTGGAAGAA	AGTTTGCCAG
		2051				2100
	mouseEGR1	GAGTGATGAA	CGCAAGAGGC	ATACCAAAAT	CCATTTAAGA	CAGAAGGACA
	ratEGR1	TTCTCTTGCC	TCCCAGAGCC	CACCCCTGAG	CTGTGCCGTG	CCGTCCAACG
50	humanEGR1	GAGCGATGAA	CGCAAGAGGC	ATACCAAGAT	CCACTTGCGG	CAGAAGGACA
		2101				2150
	mouseEGR1	AGAAAGCAGA	CAAAAGTGTG	GTGGCCTCCC	CGGCTGC...	.CTCTTCACT
	ratEGR1	ACAGCAGTCC	CATTTACTCA	GCTGCACCCA	CCTTTCTTAC	TCCCAACACT
55	humanEGR1	AGAAAGCAGA	CAAAAGTGTT	GTGGCCTCTT	CGGCCACCTC	CTCTCTCTCT
		2151				2200
	mouseEGR1	CTCTTCTTAC	CCATCCCCAG	TGGCTACCTC
	ratEGR1	GACATTTTTC	CTGAGCCCCA	AAGCCAGGCC
60	humanEGR1	TCCTACCCGT	CCCCGGTTGC	TACCTCTTAC	CCGTCCCCGG	TTACTACCTC

		2201				2250
	mouseEGR1	CTACCCATCC	CCTGCCACCA	CCTCATTTCCC	ATCCCCTGTG	CCCAC TTCCT
	rateEGR1	TTTCCTGGCT	CTGCAGGCAC	AGCCTTGCAG	TACCCGCCTC	CTGCC TACCC
5	humanEGR1	TTATCCATCC	CCGGCCACCA	CCTCATATCCC	ATCCCCTGTG	CCCAC TTCCT
		2251				2300
	mouseEGR1	ACTCCTCTCC	TGGCTCCTCC	ACCTACCCAT	CTCCTGCGCA	CAGTGGCTTC
	rateEGR1	TGCCACCAAG	GGTGGTTTCC	AGGTTCCCAT	GATCCCTGAC	TATCTGTTTC
10	humanEGR1	TCTCCTCTCC	CGGCTCCTCG	ACCTACCCAT	CCCCTGTGCA	CAGTGGCTTC
		2301				2350
	mouseEGR1	CCGTCGCCGT	CAGTGGCCAC	CACCTTTGCC	TCCGTTCC..
	rateEGR1	CACAACAACA	GGGAGACCTG	AGCCTGGGCA	CCCCAGACCA	GAAGCCCTTC
15	humanEGR1	CCCTCCCCGT	CGGTGGCCAC	CACGTACTCC	TCTGTTCCC.
		2351				2400
	mouseEGR1	...ACCTGC	TTTCCCCACC	CAGGTCAGCA	GCTTCCCGTC	TGCGGGCGTC
	rateEGR1	CAGGGTCTGG	AGAACCCTAC	CCAGCAGCCT	TCGCTCACTC	CACTATCCAC
20	humanEGR1CCTGC	TTTCCCGGCC	CAGGTCAGCA	GCTTCCCTTC	CTCAGCTGTC
		2401				2450
	mouseEGR1	AGCAGCTCCT	TCAGCACCTC	AACTGGTCTT	TCAGACATGA	CAGCGACCTT
	rateEGR1	TATCAAAGCC	TTCGCCACTC	AGTCGGGCTC	CCAGGACTTA	AAGGCTCTTA
25	humanEGR1	ACCAACTCCT	TCAGCGCCTC	CACAGGGCTT	TCGGACATGA	CAGCAACCTT
		2451				2500
	mouseEGR1	TTCTCCCAGG	ACAATTGAAA	TTTGCTAAAG	GGA.....	.ATAAAAG..
	rateEGR1	ATAACACCTA	CCAGTCCCAA	CTCATCAAAC	CCAGCCGCAT	GCGCAAGT..
30	humanEGR1	TTCTCCCAGG	ACAATTGAAA	TTTGCTAAAG	GGAAAGGGGA	AAGAAAGGGA
		2501				2550
	mouseEGR1	.AAAGCAAAG	GGAGAGGCAG	GAAAGACATA	AAAGCA...C	AGGAGGGAAG
	rateEGR1	.ACCCCAACC	GGCCCAGCAA	GACACCCCCC	CATGAACGCC	CGTATGCTTG
35	humanEGR1	AAAGGGAGAA	AAAGAAACAC	AAGAGACTTA	AAGGACAGGA	GGAGGAGATG
		2551				2600
	mouseEGR1	AGATGGCCGC	AAGAGGGGCC	ACCTCTTAGG	TCAGATGGAA	GATCTCAGAG
	rateEGR1	CCCTGTTGAG	TCCTGCGATC	GCCGCTTTTC	TCGCTCGGAT	GAGCTTACAC
40	humanEGR1	GCCATAGGAG	AGGAGGGTT.	.CCTCTTAGG	TCAGATGGAG	GTTCTCAGAG
		2601				2650
	mouseEGR1	CCAAGTCCTT	CTACTCACGA	GTA..GAAGG	ACCGTTGGCC	AACAGCCCTT
	rateEGR1	GCCACATCCG	CATCCATACA	GGC..CAGAA	GCCCTTCCAG	TGTCGAATCT
45	humanEGR1	CCAAGTCCTC	CCTCTCTACT	GGAGTGGAAG	GTCTATTGGC	CAACAATCCT
		2651				2700
	mouseEGR1	TCACTTACCA	TCCCTGCCTC	CCCCGTCCTG	TTCCCTTTGA	CTTCAGCTGC
	rateEGR1	GCATGCGTAA	TTTCAGTCGT	AGTGACCACC	TTACCACCCA	CATCCGCACC
50	humanEGR1	TTCTGCCCCAC	TTCCCCCTCC	CCAATTACTA	TTCCCTTTGA	CTTCAGCTGC
		2701				2750
	mouseEGR1	CTGAAACAGC	CATGTCCAAG	TTCTTCACCT	CTATCCAAAG	GACTTGATTT
	rateEGR1	C..ACACAGG	CGAGAAAGCCT	TTTGCTGTG	ACATTTGTGG	GAGAAAATTT
55	humanEGR1	CTGAAACAGC	CATGTCCAAG	TTCTTCACCT	CTATCCAAAG	AACTTGATTT
		2751				2800
	mouseEGR1	GCATGG....	..TATTGGAT	AAATCATTTT	AGTATCCTCT
	rateEGR1	GCCAGGAGTG	ATGAACGCAA	GAGGCATACC	AAAATCCACT	TAAGACAGAA
60	humanEGR1	GCATGGA...	..TTTTGGAT	AAATCATTTT	AGTATCATCT

		2801				2850
	mouseEGR1CCATC	ACATGCCTGG	CCCTTGCTCC	CTTCAGCGCT	AGACCATCAA
	ratEGR1	GGACAAGAAA	GCAGACAAAA	GTGTCGTGGC	CTCCTCAGCT	GCCTCTTCCC
5	humanEGR1CCATCA	TATGCCTGAC	CCCTTGCTCC	CTTCAATGCT	AGAAAAATCGA
		2851				2900
	mouseEGR1	GTTGGCATAA	AGAAAAAAAA	ATGGGTTTGG	GCCCTCAGAA	CCCTGCCCTG
	ratEGR1	TCTCTTCCTA	CCCATCCCCA	GTGGCTACCT	CCTACCCATC	CCCCGCCACC
10	humanEGR1	GTTGGC....AAAT	GGGGTTTGGG	CCCCTCAGAG	CCCTGCCCTG
		2901				2950
	mouseEGR1	CATCTTTGTA	CAGCATCTGT	GCCATGGATT	TTGTTTTTCCT	TGGGGTATTC
	ratEGR1	ACCTCATTTT	CATCCCCAGT	GCCCCACCTCT	TACTCCTCTC	CGGGCTCCTC
15	humanEGR1	CACCCTTGTA	CAGTGTCTGT	GCCATGGATT	TCGTTTTTCT	TGGGGTACTC
		2951				3000
	mouseEGR1	TTGATGTGAA	GATAATTTGC	ATACT.....	.CTATTGTAT	TATTTGGAGT
	ratEGR1	TACCTACCCG	TCTCCTGCAC	ACAGTGGCTT	CCCATCGCCC	TCGGTGGCCA
20	humanEGR1	TTGATGTGAA	GATAATTTGC	ATATT.....	.CTATTGTAT	TATTTGGAGT
		3001				3050
	mouseEGR1	TAAATCCTCA	CTTTGGGG..	GAGGGGGGAG	CAAAGCCAAG	CAAACCAATG
	ratEGR1	CCACCTATGC	CTCCGTCC..	CACCTGCTTT	CCCTGCCCAG	GTGAGCACCT
25	humanEGR1	TAGGTCCCTCA	CTTGGGGGAA	AAAAAAAAAA	AAAAGCCAAG	CAAACCAATG
		3051				3100
	mouseEGR1	ATGATCCTCT	ATTTTGTGAT	GACTCTGCTG	TGACATTA..
	ratEGR1	TCCAGTCTGC	AGGGGTCAGC	AACTCCTTCA	GCACCTCAAC	GGGTCTTTCA
30	humanEGR1	GTGATCCTCT	ATTTTGTGAT	GATGCTGTGA	CAATA.....
		3101				3150
	mouseEGR1	.GGTTTGAAG	CATTTTTTTTT	TTCAAGCAGC	AGTCCTAGGT	ATTAACCTGGA
	ratEGR1	GACATGACAG	CAACCTTTTT	TCCTAGGACA	ATTGAAATTT	GCTAAAGGGA
35	humanEGR1	...AGTTTGA	ACCTTTTTTTT	TTGAAACAGC	AGTCCCAG..	..TATTCTCA
		3151				3200
	mouseEGR1	..GCATGTGT	CAGAGTGTTG	TTCCGTTAAT	TTTGTAATAA	CTGGCTCGAC
	ratEGR1	ATGAAAGAGA	GCAAAGGGAG	GGGAGCGCGA	GAGACAATAA	AGGACAGGAG
40	humanEGR1	GAGCATGTGT	CAGAGTGTTG	TTCCGTTAAC	CTTTTTGTAA	ATACTGCTTG
		3201				3250
	mouseEGR1	.TGTAACCTCT	CACATGTGAC	AAAGTATGGT	TTGTTTG GTT	GGGTTTTTGT
	ratEGR1	.GGAAGAAAT	GGCCCGCAAG	AGGGGCTGCC	TCTTAGGTCA	GATGGAAGAT
45	humanEGR1	ACCGTACTCT	CACATGTGGC	AAAATATGGT	TTGGTTTTTC	TTTTTTTTTTT
		3251				3300
	mouseEGR1	TTTGAGAAAT	TTTTTGCCCC	TCCCTTTGGT	TTCAAAAAGTT	TCACGTCTTG
	ratEGR1	CTCAGAGCCA	AGTCCTTCTA	GTCAGTAGAA	GGCCCGTTGG	CCACCAGCCC
50	humanEGR1	TTGAAAGTGT	TTTTTCTTCG	TCCTTTTGGT	TTAAAAAGTT	TCACGTCTTG
		3301				3350
	mouseEGR1	GTGCCTTTTG	TGTGACACGC	CTT.CCGATG	GCTTGACATG	CGCA.....
	ratEGR1	TTTCACTTAG	CGTCCCCTGCC	CTC.CCCAGT	CCCGGTCCCT	TTGACTTCAG
55	humanEGR1	GTGCCTTTTG	TGTGATGCCC	CTTGCTGATG	GCTTGACATG	TGCAAT....
		3351				3400
	mouseEGR1	...GATGTGA	GGGACACGCT	CACCTTAGCC	TTAA...GGG	GGTAGGAGTG
	ratEGR1	CTGCCTGAAA	CAGCCACGTC	CAAGTTCTTC	ACCT...CTA	TCCAAAGGAC
60	humanEGR1TGTGA	GGGACATGCT	CACCTCTAGC	CTTAAGGGGG	GCAGGGAGTG

		3401				3450
	mouseEGR1	ATGTGTTGGG	GGAGGCTTGA	GAGCAAAAAC	GAGGAAGAGG	GCTGAGCTGA
	rateEGR1	TTGATTTGCA	TGGTATTGGA	TAAACCATTT	CAGCATCATC	TCCACCACAT
5	humanEGR1	ATGATTTGGG	GGAGGCTTTG	GGAGCAAAAT	AAGGAAGAGG	GCTGAGCTGA
		3451				3500
	mouseEGR1	GCTTTCGGTC	TCCAGAATGT	AAGAAGAAAA	AATTTAAACA	AAAATCTGAA
	rateEGR1	GCCTGGCCCT	TGCTCCCTTC	AGCACTAGAA	CATCAAGTTG	GCTGAAAAAA
10	humanEGR1	GCTTCGGTTC	TCCAGAATGT	AAGAAAACAA	AATCTAAAC	AAAATCTGAA
		3501				3550
	mouseEGR1	CTCTCAAAAG	TCTATTTTTTC	TAAACTGAAA	ATGTAAATTT	ATACATCTAT
	rateEGR1	AAAATGGGTC	TGGGCCCTCA	GAACCCTGCC	CTGTATCTTT	GTACA.....
15	humanEGR1	CTCTCAAAAG	TCTATTTTTTT	TAA.CTGAAA	ATGTAAATTT	ATAAATATAT
		3551				3600
	mouseEGR1	TCAGGAGTTG	GAGTGTGTGTG	GTTACCTACT	GAGTAGGCTG	CAGTTTTTTGT
	rateEGR1	GCATCTGTGC	CATGGATTTT	GTTTTCTTTG	GGGTATTCTT	GATGTGAAGA
20	humanEGR1	TCAGGAGTTG	GAATGTTGTA	GTTACCTACT	GAGTAGGCGG	CGATTTTTTGT
		3601				3650
	mouseEGR1	ATGTTATGAA	CATGAAGTTC	ATTATTTTGT	GGTTTTATTT	TACTTTGTAC
	rateEGR1	TAATTTGCAT	ACTCTATTGT	ACTATTTGGA	GTTAAATTCT	CACTTTGGGG
25	humanEGR1	ATGTTATGAA	CATGCAGTTC	ATTATTTTGT	GGTTCTATTT	TACTTTGTAC
		3651				3700
	mouseEGR1	TTGTGTTTGC	TTAAACAAAG	TAACCTGTTT	GGCTTATAAA	CACATTGAAT
	rateEGR1	GAGGGGGAGC	AAAGCCAAGC	AAACCAATGG	TGATCCTCTA	TTTTGTGATG
30	humanEGR1	TTGTGTTTGC	TTAAACAAAG	TGA.CTGTTT	GGCTTATAAA	CACATTGAAT
		3701				3750
	mouseEGR1	GCGCTCTATT	GCCCATGG..	..GATATGTG	GTGTGTATCC	TTCAGAAAAA
	rateEGR1	ATCCTGCTGT	GACATTAGGT	TTGAAACTTT	TTTTTTTTTT	TGAAGCAGCA
35	humanEGR1	GCGCTTTATT	GCCCATGG..	..GATATGTG	GTGTATATCC	TTCCAAAAAA
		3751				3800
	mouseEGR1	TTAAAAGGAA	AAAT.....
	rateEGR1	GTCCTAGGTA	TTAACCTGGAG	CATGTGTCAG	AGTGTGTTC	CGTTAATTTT
40	humanEGR1	TTAAAACGAA	AATAAAGTAG	CTGCGATTGG	G.....
		3801				3850
	mouseEGR1
	rateEGR1	GTAAATACTG	CTCGACTGTA	ACTCTCACAT	GTGACAAAAT	ACGGTTTGT
45	humanEGR1
		3851				3900
	mouseEGR1
	rateEGR1	TGGTTGGGTT	TTTTGTGTGT	TTTGAAAAAA	AAATTTTTTT	TTGCCCCGTC
50	humanEGR1
		3901				3950
	mouseEGR1
	rateEGR1	CCTTTGGT	CAAAAGTTTC	ACGTCTTGGT	GCCTTTGTGT	GACACACCTT
55	humanEGR1
		3951				4000
	mouseEGR1
	rateEGR1	GCCGATGGCT	GGACATGTGC	AATCGTGAGG	GGACACGCTC	ACCTCTAGCC
60	humanEGR1

		4001				4050
	mouseEGR1
	ratEGR1	TTAAGGGGGT	AGGAGTGATG	TTTCAGGGGA	GGCTTTAGAG	CACGATGAGG
5	humanEGR1
		4051				4100
	mouseEGR1
	ratEGR1	AAGAGGGCTG	AGCTGAGCTT	TGGTTCTCCA	GAATGTAAGA	AGAAAAATTT
10	humanEGR1
		4101				4150
	mouseEGR1
	ratEGR1	AAAACAAAAA	TCTGAACTCT	CAAAAGTCTA	TTTTTTTAAC	TGAAAATGTA
15	humanEGR1
		4151				4200
	mouseEGR1
	ratEGR1	GATTTATCCA	TGTTCTGGGAG	TTGGAATGCT	GCGGTTACCT	ACTGAGTAGG
20	humanEGR1
		4201				4250
	mouseEGR1
	ratEGR1	CGGTGACTTT	TGTATGCTAT	GAACATGAAG	TTCATTATTT	TGTGGTTTTTA
25	humanEGR1
		4251				4300
	mouseEGR1
	ratEGR1	TTTTFACTTCG	TACTTGTGTT	TGCTTAAACA	AAGTGACTTG	TTTGGCTTAT
30	humanEGR1
		4301				4350
	mouseEGR1
	ratEGR1	AAACACATTG	AATGCGCTTT	ACTGCCCATG	GGATATGTGG	TGTGTATCCT
35	humanEGR1
		4351			4388	
	mouseEGR1	
	ratEGR1	TCAGAAAAAT	TAAAAGGAAA	ATAAAGAAAC	TAACTGGT	
40	humanEGR1	

EXPERIMENTAL DETAILS

EXAMPLE 1

5 **Role of EGR-1 in endothelial cell proliferation and migration**

Materials and Methods

10 *Oligonucleotides and chemicals.* Phosphorothioate-linked antisense oligonucleotides directed against the region comprising the translational start site of Egr-1 mRNA were synthesized commercially (Genset Pacific) and purified by high performance liquid chromatography. The target sequence of AS2 (5'-CsTsTsGsGsCsGsCsTsGsCsCsAsT-3') (SEQ ID NO:16) is conserved in mouse, rat and human Egr-1 mRNA. For control purposes, we used AS2C
15 (5'-GsCsAsCsTsTsCsTsGsCsTsGsTsCsC-3') (SEQ ID NO:17), a size-matched phosphorothioate-linked counterpart of AS2 with similar base composition. Phorbol-12-myristate 13-acetate (PMA) and fibroblast growth factor-2 were purchased from Sigma-Aldrich.

20 *Cell culture.* Bovine aortic endothelial cells were obtained from Cell Applications, Inc. and used between passages 5-9. The endothelial cells were grown in Dulbecco's modified Eagles' medium (Life Technologies), pH 7.4, containing 10% fetal bovine serum supplemented with 50 µg/mL streptomycin and 50 IU/mL penicillin. The cells were routinely passaged with trypsin/EDTA and maintained at 37°C in a humidified atmosphere of 5%
25 CO₂/95% air.

Transient transfection analysis and CAT assay. The endothelial cells were grown to 60-70% confluence in 100mm dishes and transiently transfected with 10 µg of the indicated chloramphenicol acetyl transferase (CAT)-based promoter reporter construct using FuGENE6 (Roche). The cells
30 were rendered growth-quiescent by incubation 48 h in 0.25% FBS, and stimulated with various agonists for 24 h prior to harvest and assessment of CAT activity. CAT activity was measured and normalized to the concentration of protein in the lysates (determined by Biorad Protein Assay) as previously described (Khachigian et al., 1999).

35 *Northern blot analysis.* Total RNA (12 µg/well) of growth-arrested endothelial cells (prepared using TRIzol Reagent (Life Technologies) in

accordance with the manufacturer's instructions) previously exposed to various agonists for 1 h was resolved by electrophoresis on denaturing 1% agarose-formaldehyde gels. Following transfer overnight to Hybond- N+ nylon membranes (Amersham), the blots were hybridized with ³²P-labeled
5 Egr-1 cDNA prepared using the Nick Translation Kit overnight (Roche). The membranes were washed and radioactivity visualized by autoradiography as previously described (Khachigian et al., 1995).

RT-PCR. Reverse transcription was performed with 8 µg of total RNA using M-MLV reverse transcriptase. Egr-1 cDNA was amplified (334 bp
10 product (Delbridge et al., 1997)) using Taq polymerase by heating for 1 min at 94°C, and cycling through 94°C for 1 min, 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following thirty cycles, a 5 min extension at 72°C was carried out. Samples were electrophoresed on 1.5% agarose gel containing ethidium bromide and photographed under ultraviolet illumination. β-actin
15 amplification (690 bp product) was performed essentially as above. The sequences of the primers were: Egr-1 forward primer (5'-GCA CCC AAC AGT GGC AAC-3') (SEQ ID NO:18), Egr-1 reverse primer (5'-GGG ATC ATG GGA ACC TGG-3') (SEQ ID NO:19), β-actin forward primer (5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3') (SEQ ID NO:20), and β-actin reverse primer
20 (5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3') (SEQ ID NO:21).

Antisense oligonucleotide delivery and Western blot analysis. Growth-arrested cells in 100 mm dishes were incubated with the indicated oligonucleotides 24 h and 48 h after the initial change of medium. When oligonucleotide was added a second time, the cells were incubated with
25 various concentrations of insulin and harvest 1 h subsequently. The cells were washed in cold phosphate-buffered saline (PBS), pH 7.4, and solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 µg/ml leupeptin, 1% aprotinin, 2 µM PMSF). Lysates were resolved by
30 electrophoresis on 8% denaturing SDS-polyacrylamide gels, transferred to PDVF nylon membranes (NEN-DuPont), blocked with skim milk powder, then incubated with polyclonal antibodies to Egr-1 (Santa Cruz Biotechnology, Inc) and monoclonal horseradish peroxidase-linked mouse anti-rabbit Ig secondary antibodies followed by chemiluminescent detection
35 (NEN-DuPont).

³H-Thymidine incorporation into DNA. Growth-arrested endothelial cells at 90% confluence in 96 well plates were incubated twice with the oligonucleotides prior to the addition of insulin. When signaling inhibitors (PD98059, SB202190, wortmannin) were used in experiments, these agents were added 2 h before the addition of insulin. After 18 h of exposure to insulin, the cells were pulsed with 200,000 cpm/well of methyl-³H thymidine (NEN-DuPont) for 6 h. Lysates were prepared by washing first in cold PBS, pH7.4, then fixing with cold 10% trichloroacetic acid, washing with cold ethanol and solubilizing in 0.1 M NaOH. ³H-Thymidine in the lysates was quantitated with ACSII scintillant using β -scintillation counter (Packard).

In vitro injury. Growth-arrested cells at 90% confluence were incubated with antisense oligonucleotides and insulin at various concentrations as described above, then were scraped by drawing a sterile wooden toothpick across the monolayer (Khachigian et al., 1996). Following 48-72 h, the cells were fixed in 4% formalin, stained with hematoxylin/eosin then photographed.

HMEC-1 culture and proliferation assay. SV40-transformed HMEC-1 cells were grown in MCDB 131 medium with EGF (10 ng/ml) and hydrocortisone (1 μ g/ml) supplements and 10% FBS. Forty-eight h after incubation in serum-free medium without supplements, the cells were transfected with the indicated DNA enzyme (0.4 μ M) and transfected again 72 h after the change of medium, when 10% serum was added. The cells were quantitated by Coulter counter, 24 h after the addition of serum.

Antisense Egr-1 mRNA overexpression. Bovine aortic endothelial cells or rat vascular smooth muscle cells were grown to 60% confluence in 96-well plates then transfected with 3 μ g of construct pcDNA3-A/SEgr-1 (in which a 137bp fragment of Egr-1 cDNA (732-869) was cloned in antisense orientation into the BamHI/EcoRI site of pcDNA3), or pcDNA3 alone, using Fugene6 in accordance with the manufacturer's instructions. Growth arrested cells were incubated with 5% FBS in Waymouth's medium (SMC) or DMEM (EC) and trypsinised after 3 days prior to quantitation of the cell populations by Coulter counting.

Results and Discussion

Insulin, but not Glucose, Stimulates Egr-1 Activity in Vascular Endothelial Cells. High glucose may activate normally-quiescent vascular

endothelium by stimulating mitogen-activated protein (MAP) kinase activity and the expression of immediate-early genes (Frodin et al., 1995; Kang et al., 1999). These signaling and transcriptional events may, in turn, induce the expression of other genes whose products then alter endothelial phenotype and facilitate the development of lesions. To determine the effect of glucose on Egr-1 activity in vascular endothelial cells, we performed transient transfection analysis in endothelial cells transfected with pEBS1³foscet, a chloramphenicol acetyltransferase (CAT)-based reporter vector driven by three high-affinity Egr-1 binding sites placed upstream of the c-fos TATA box (Gashler et al., 1993). Exposure of growth-arrested endothelial cells to various concentrations of glucose (5 to 30 mM) over 24 h did not increase Egr-1 binding activity (Figure 1). However, Egr-1 binding activity did increase in cells exposed to insulin (100 nM) (Figure 1). Reporter activity also increased upon incubation with FGF-2, a known inducer of Egr-1 transcription and binding activity in vascular endothelial cells (Santiago et al., 1999b) (Figure 1).

Insulin and FGF-2 Induce Egr-1 mRNA Expression in Vascular Endothelial Cells. The preceding findings using reporter gene analysis provided evidence for increased Egr-1 expression in endothelial cells exposed to insulin. We next used reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis to demonstrate directly the capacity of insulin to increase levels of Egr-1 mRNA. RT-PCR revealed that Egr-1 is weakly expressed in growth-quiescent endothelial cells (data not shown). Insulin, like FGF-2, increased Egr-1 expression within 1 h of exposure to the agonist. In contrast, levels of β -actin mRNA were unchanged. Northern blot analysis confirmed these qualitative data by demonstrating that insulin, FGF-2, and phorbol 12-myristate 13-acetate (PMA), a second potent inducer of Egr-1 expression (Khachigian et al., 1995) elevated steady-state Egr-1 mRNA levels within 1 h without increasing levels of ribosomal 28S and 18S mRNA (data not shown).

Insulin-Stimulated Egr-1 Protein Synthesis in Endothelial Cells is Inhibited by Antisense Oligonucleotides Targeting Egr-1 mRNA. To reconcile our demonstration of insulin-induced Egr-1 mRNA expression with the binding activity of the transcription factor (Figure 1), we performed Western immunoblot analysis using polyclonal antibodies directed against Egr-1 protein. Insulin (at 100 nM and 500 nM) induced Egr-1 protein synthesis in

growth-arrested endothelial cells within 1 h (data not shown). These findings, taken together, demonstrate that insulin elevates Egr-1 mRNA, protein and binding activity in vascular endothelial cells.

We recently developed phosphorothioate-based antisense
5 oligonucleotides targeting the translational start site in Egr-1 mRNA (Santiago et al., 1999c). These oligonucleotides lack phosphorothioate G-quartet sequences that have been associated with non-specific biological activity (Stein, 1997). Western blot analysis revealed that prior incubation of growth-arrested endothelial cells with 0.8 μ M antisense Egr-1
10 oligonucleotides (AS2) inhibited insulin-inducible Egr-1 protein synthesis, despite equal loading of protein. The lack of attenuation in insulin-inducible Egr-1 protein following exposure of the cells to an identical concentration of AS2C demonstrates the sequence-specific inhibitory effect of the antisense Egr-1 oligonucleotides.

15 *Insulin Stimulates Endothelial Cell DNA Synthesis which is Inhibited by Antisense Oligonucleotides Targeting Egr-1 mRNA.* These oligonucleotides, which attenuate the induction of Egr-1 protein, were used in 3 H-thymidine incorporation assays to determine the involvement of Egr-1 in insulin-inducible DNA synthesis. This assay evaluates 3 H-thymidine uptake into
20 DNA precipitable with trichloroacetic acid (TCA) (Khachigian et al., 1992). In initial experiments, growth-arrested endothelial cells exposed to insulin (100 nM) increased the extent of DNA synthesis by 100%, whereas 500 nM insulin caused a 200% increase in DNA synthesis (Figure 2A).

We next determined the effect of AS2 and AS2C on insulin-inducible
25 endothelial DNA synthesis. In the absence of added insulin, AS2 (0.8 μ M) inhibited basal endothelial DNA synthesis facilitated by low concentrations of serum (0.25% v:v) (Figure 2B). In contrast, the scrambled control (0.8 μ M) or a third oligonucleotide, E3 (0.8 μ M), a size-matched phosphorothioate directed toward another region of Egr-1 mRNA (Santiago et al., 1999c) had
30 little effect on basal DNA synthesis (Figure 2B). Furthermore, unlike AS2 and E3, AS2 significantly inhibited DNA synthesis inducible by insulin (500 nM and 1000 nM) (Figure 2B). To demonstrate concentration-dependent inhibition of DNA synthesis, we incubated the endothelial cells with 0.4 μ M as well as 0.8 μ M of Egr-1 oligonucleotide. Since this lower concentration of
35 AS2 inhibited 3 H-thymidine incorporation less effectively (compare to AS2C) indicates dose-dependent and sequence-specific inhibition by the antisense

Egr-1 oligonucleotide (Figure 2C). These findings thus demonstrate the requirement for Egr-1 protein in endothelial cell DNA synthesis inducible by insulin.

Insulin-Stimulated DNA Synthesis in Endothelial Cells is Inhibited by PD98059 and Wortmannin, But Not by SB202190. Inducible Egr-1 transcription is governed by the activity of extracellular signal-regulated kinase (ERK) (Santiago et al., 1999b) which phosphorylates factors at serum response elements in the Egr-1 promoter (Gashler et al, 1995). Since there is little known about signaling pathways mediating insulin-inducible proliferation of vascular endothelial cells, we determined the relevance of MEK/ERK in this process using the specific MEK/ERK inhibitor, PD98059. This compound (at 10 and 30 μ M) inhibited insulin-inducible DNA synthesis in a dose-dependent manner (Figure 3). Likewise, wortmannin (0.3 and 1 μ M), the phosphatidylinositol 3-kinase inhibitor which also inhibits c-Jun N-terminal kinase (JNK) (Ishizuka et al, 1999; Day et al., 1999; Kumahara et al., 1999), ERK (Barry et al., 1999) and p38 kinase (Barry et al., 1999) inhibited DNA synthesis in a dose-dependent manner (Figure 3). In contrast, SB202190 (100 and 500 nM), a specific p38 kinase inhibitor failed to affect DNA synthesis (Figure 3). These findings demonstrate the critical role for MEK/ERK, and possibly JNK, in insulin-inducible endothelial cell proliferation, and the lack of p38 kinase involvement in this process.

Insulin Stimulates Endothelial Cell Regrowth After Mechanical Injury In Vitro in an Egr-1-Dependent Manner. Mechanically wounding vascular endothelial (and smooth muscle) cells in culture results in migration and proliferation at the wound edge and the eventual recoverage of the denuded area. We hypothesized that insulin would accelerate this cellular response to mechanical injury. Acutely scraping the growth-quiescent (rendered by 48 h incubation in 0.25% serum) endothelial monolayer resulted in a distinct wound edge (data not shown). Continued incubation of the cultures in medium containing low serum for a further 3 days resulted in weak regrowth in the denuded zone but aggressive regrowth in the presence of optimal amounts of serum (10%). When insulin (500 nM) was added to growth-quiescent cultures at the time of injury the population of cells in the denuded zone significantly increased, albeit as expected, less efficiently than the 10% serum control.

To investigate the involvement of Egr-1 in endothelial regrowth potentiated by insulin after injury we incubated the cultures with antisense Egr-1 oligonucleotides prior to scraping and again at the time of injury and the addition of insulin. AS2 (0.8 μ M) significantly inhibited endothelial regrowth stimulated by insulin. In contrast, regrowth in the presence of AS2C (0.8 μ M) was not significantly different from cultures in which oligonucleotide was omitted. Similar findings were observed when higher concentrations (1.2 μ M) of AS2 and AS2C were used. Thus, endothelial regrowth after injury stimulated by insulin proceeds in an Egr-1-dependent manner. These observations are quantitated in Figure 4.

These results show that insulin-induced proliferation and regrowth after injury are processes critically dependent upon the activation of Egr-1. Northern blot, RT-PCR and Western immunoblot analysis reveal that insulin induces Egr-1 mRNA and protein expression. Antisense oligonucleotides which block insulin-induced synthesis of Egr-1 protein in a sequence-specific and dose-dependent manner, also inhibit proliferation and regrowth after mechanical injury. These findings using nucleic acids specifically targeting Egr-1 demonstrate the functional involvement of this transcription factor in endothelial growth.

Insulin signaling involves the activation of a growing number of immediate-early genes and transcription factors. These include c-fos (Mohn et al., 1990; Jhun et al, 1995; Harada et al., 1996), c-jun (Mohn et al., 1990), nuclear factor- κ B (Bertrand et al., 1998), SOCS3 (Emanuelli et al., 2000) and the forkhead transcription factor FKHR (Nakae et al., 1999). Insulin also induces the expression of Egr-1 in mesangial cells (Solow et al., 1999), fibroblasts (Jhun et al., 1995), adipocytes (Alexander-Bridges et al., 1992) and Chinese hamster ovary cells (Harada et al., 1996). This study is the first to describe the induction of Egr-1 by insulin in vascular endothelial cells.

Insulin activates several subclasses within the MAP kinase superfamily, including ERK, JNK and p38 kinase (Guo et al., 1998). Our findings indicate that the specific ERK inhibitor PD98059, which binds to MEK and prevents phosphorylation by Raf, inhibits insulin-inducible endothelial cell proliferation. Egr-1 transcription is itself dependent upon the phosphorylation activity of ERK via its activation of ternary complex factors (such as Elk-1) at serum response elements (SRE) in the Egr-1 promoter. Six SREs appear in the Egr-1 promoter whereas only one is present

in the c-fos promoter (Gashler et al., 1995). PD98059 blocks insulin-inducible Elk-1 transcriptional activity at the c-fos SRE in vascular cells (Xi et al., 1997). These published findings are consistent with the present demonstration of the involvement of Egr-1 in insulin-inducible proliferation.

5 To provide evidence, independent of insulin, that endothelial proliferation is an Egr-1-dependent process, we incubated human microvascular endothelial cells (HMEC-1) separately with two DNA enzymes (DzA and DzF) each targeting different sites in human EGR-1 mRNA, at a final concentration of 0.4 μ M. DzA and DzF both inhibited HMEC-1
10 replication (total cell counts) in the presence of 5% serum (Figure 5). In contrast, DzFscr, was unable to modulate proliferation at the same concentration (Figure 5). DzFscr bears the same active 15nt catalytic domain as DzF and has the same net charge but has scrambled hybridizing arms. These data obtained using a second endothelial cell type demonstrate
15 inhibition of endothelial proliferation using sequence-specific strategies targeting human EGR-1.

Finally, we found that CMV-mediated overexpression of antisense Egr-1 mRNA inhibited proliferation of both endothelial cells and smooth muscle cells. Replication of both endothelial and smooth muscle cell pcDNA3-
20 A/SEgr-1 transfectants was significantly lower than those transfected with the backbone vector alone, pcDNA3 (data not shown). These findings demonstrate that antisense EGR mRNA strategies can inhibit proliferation of arterial endothelial cells and at least one other vascular cell type.

Despite the availability and clinical use of a large number of
25 chemotherapeutic agents for the clinical management of neoplasia, solid tumours remain a major cause of mortality in the Western world. Drugs currently used to treat such tumours are generally non-specific poisons that can be toxic to non-cancerous tissue and require high doses for efficacy. There is growing evidence that the cellular and molecular mechanisms
30 underlying tumour growth involves more than just tumour cell proliferation and migration. Importantly, tumour growth and metastasis are critically dependent upon ongoing angiogenesis, the process new blood vessel formation (Crystal et al., 1999). The present findings, which demonstrate that Egr-1 is critical in vascular endothelial cell replication and migration,
35 strongly implicate this transcription factor as a key regulator in angiogenesis and tumorigenesis.

Example 2

Characterisation of DNAzyme targeting rat Egr-1 (NGFI-A)

5 Materials and Methods

ODN synthesis. DNAzymes were synthesized commercially (Oligos Etc., Inc.) with an inverted T at the 3' position unless otherwise indicated. Substrates in cleavage reactions were synthesized with no such modification. Where indicated ODNs were 5'-end labeled with $\gamma^{32}\text{P}$ -dATP and T4
10 polynucleotide kinase (New England Biolabs). Unincorporated label was separated from radiolabeled species by centrifugation on Chromaspin-10 columns (Clontech).

In vitro transcript and cleavage experiments. A ^{32}P -labelled 206 nt NGFI-A RNA transcript was prepared by in vitro transcription (T3
15 polymerase) of plasmid construct pJDM8 (as described in Milbrandt, 1987, the entire contents of which are incorporated herein by reference) previously cut with *Bgl* II. Reactions were performed in a total volume of 20 μl containing 10 mM MgCl_2 , 5 mM Tris pH 7.5, 150 mM NaCl, 4.8 pmol of in vitro transcribed or synthetic RNA substrate and 60 pmol DNAzyme (1:12.5
20 substrate to DNAzyme ratio), unless otherwise indicated. Reactions were allowed to proceed at 37 °C for the times indicated and quenched by transferring an aliquot to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were run on 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

Culture conditions and DNAzyme transfection. Primary rat aortic SMCs were obtained from Cell Applications, Inc., and grown in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 50 $\mu\text{g}/\text{ml}$ streptomycin and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2 . SMCs were used in experiments between passages 3-7. Pup rat
30 SMCs (WKY12-22 (as described in Lemire et al, 1994, the entire contents of which are incorporated herein by reference)) were grown under similar conditions. Subconfluent (60-70%) SMCs were incubated in serum-free medium (SFM) for 6 h prior to DNAzyme (or antisense ODN, where indicated) transfection (0.1 μM) using Superfect in accordance with
35 manufacturer's instructions (Qiagen). After 18 h, the cells were washed with

phosphate-buffered saline (PBS), pH 7.4 prior to transfection a second time in 5% FBS.

Northern blot analysis. Total RNA was isolated using the TRIzol reagent (Life Technologies) and 25 μ g was resolved by electrophoresis prior to transfer to Hybond-N+ membranes (NEN-DuPont). Prehybridization, hybridization with α^{32} P-dCTP-labeled Egr-1 or β -Actin cDNA, and washing was performed essentially as previously described (Khachigian et al, 1995).

Western blot analysis. Growth-quiescent SMCs in 100 mm plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, and incubated with 5% FBS for 1 h. The cells were washed in cold PBS, pH 7.4, and extracted in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 μ g/ml leupeptin, 1% aprotinin and 2 mM PMSF. Twenty four μ g protein samples were loaded onto 10% denaturing SDS-polyacrylamide gels and electroblotted onto PVDF nylon membranes (NEN-DuPont). Membranes were air dried prior to blocking with non-fat skim milk powder in PBS containing 0.05% (w:v) Tween 20. Membranes were incubated with rabbit antibodies to Egr-1 or Sp1 (Santa Cruz Biotechnology, Inc.) (1:1000) then with HRP-linked mouse anti-rabbit Ig secondary antiserum (1:2000). Where mouse monoclonal c-Fos (Santa Cruz Biotechnology, Inc.) was used, detection was achieved with HRP-linked rabbit anti-mouse Ig. Proteins were visualized by chemiluminescent detection (NEN-DuPont).

Assays of cell proliferation. Growth-quiescent SMCs in 96-well titer plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, then exposed to 5% FBS at 37 °C for 72 h. The cells were rinsed with PBS, pH 7.4, trypsinized and the suspension was quantitated using an automated Coulter counter.

Assessment of DNAzyme stability. DNAzymes were 5'-end labeled with γ^{32} P-dATP and separated from free label by centrifugation. Radiolabeled DNAzymes were incubated in 5% FBS or serum-free medium at 37 °C for the times indicated. Aliquots of the reaction were quenched by transfer to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were applied to 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

SMC wounding assay. Confluent growth-quiescent SMCs in chamber slides (Nunc-InterMed) were exposed to ED5 or ED5SCR for 18 h prior to a

single scrape with a sterile toothpick. Cells were treated with mitomycin C (Sigma) (20 μ M) for 2 h prior to injury (Pitsch et al, 1996; Horodyski & Powell, 1996). Seventy-two h after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde then stained with hematoxylin-eosin.

5 *Rat arterial ligation model and analysis.* Adult male Sprague Dawley rats weighing 300-350 g were anaesthetised using ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right common carotid artery was exposed up to the carotid bifurcation via a midline neck incision. Size 6/0 non-absorbable suture was tied around the common carotid proximal to the
10 bifurcation, ensuring cessation of blood flow distally. A 200 μ l solution at 4°C containing 500 μ g of DNAzyme (in DEPC-treated H₂O), 1mM MgCl₂, 30 μ l of transfecting agent (Fugene 6) and Pluronic gel P127 (BASF) was applied around the vessel in each group of 5 rats, extending proximally from the ligation for 12-15 mm. These agents did not inhibit the solidification of the
15 gel at 37 °C. After 3 days, vehicle with or without 500 μ g of DNAzyme was administered a second time. Animals were sacrificed 18 days after ligation by lethal injection of phenobarbitone, and perfusion fixed using 10% (v:v) formaldehyde perfused at 120 mm Hg. Both carotids were then dissected free and placed in 10% formaldehyde, cut in 2 mm lengths and embedded in 3%
20 (w:v) agarose prior to fixation in paraffin. Five μ m sections were prepared at 250 μ m intervals along the vessel from the point of ligation and stained with hematoxylin and eosin. The neointimal and medial areas of 5 consecutive sections per rat were determined digitally using a customized software package (Magellan) (Halasz & Martin, 1984) and expressed as a mean ratio
25 per group of 5 rats.

Results and Discussion

The 7x7 nt arms flanking the 15 nt DNAzyme catalytic domain in the original DNAzyme design (Santoro and Joyce, 1997) were extended by 2 nts
30 per arm for improved specificity (L.-Q. Sun, data not shown) (Figure 6). The 3' terminus of the molecule was capped with an inverted 3'-3'-linked thymidine (T) to confer resistance to 3'->5' exonuclease digestion. The sequence in both arms of ED5 was scrambled (SCR) without altering the catalytic domain to produce DNAzyme ED5SCR (Figure 6).

35 A synthetic RNA substrate comprised of 23 nts, matching nts 805 to 827 of NGFI-A mRNA (Figure 6) was used to determine whether ED5 had the

capacity to cleave target RNA. ED5 cleaved the ^{32}P -5'-end labeled 23-mer within 10 min (data not shown). The 12-mer product corresponds to the length between the A(816)-U(817) junction and the 5' end of the substrate (Figure 6). In contrast, ED5SCR had no demonstrable effect on this synthetic
5 substrate. Specific ED5 catalysis was further demonstrated by the inability of the human equivalent of this DNAzyme (hED5) to cleave the rat substrate over a wide range of stoichiometric ratios (data not shown). Similar results were obtained using ED5SCR (data not shown). hED5 differs from the rat ED5 sequence by 3 of 18 nts in its hybridizing arms (Table 2). The catalytic
10 effect of ED5 on a ^{32}P -labeled 206 nt fragment of native NGFI-A mRNA prepared by *in vitro* transcription was then determined. The cleavage reaction produced two radiolabeled species of 163 and 43 nt length consistent with DNAzyme cleavage at the A(816)-U(817) junction. In other experiments, ED5 also cleaved a ^{32}P -labeled NGFI-A transcript of 1960 nt
15 length in a specific and time-dependent manner (data not shown).

Table 2. DNAzyme target sites in mRNA.

Similarity between the 18 nt arms of ED5 or hED5 and the mRNA of rat
 5 NGFI-A or human EGR-1 (among other transcription factors) is expressed as a
 percentage. The target sequence of ED5 in NGFI-A mRNA is 5'-807-A
 CGU CCG GGA UGG CAG CGG-825-3' (SEQ ID NO: 22) (rat NGFI-A
 sequence), and that of hED5 in EGR-1 is 5'-262-U CGU CCA GGA UGG CCG
 CGG-280-3' (SEQ ID NO: 23) (Human EGR-1 sequence). Nucleotides in bold
 10 indicate mismatches between rat and human sequences. Data obtained by a
 gap best fit search in ANGIS using sequences derived from Genbank and
 EMBL. Rat sequences for Sp1 and c-Fos have not been reported.

Gene	Accession number	Best homology over 18 nts (%)	
		ED5	hED5
Rat NGFI-A	M18416	100	84.2
20 Human EGR-1	X52541	84.2	100
Murine Sp1	AF022363	66.7	66.7
Human c-Fos	K00650	66.7	66.7
Murine c-Fos	X06769	61.1	66.7
Human Sp1	AF044026	38.9	28.9
25			

To determine the effect of the DNAzymes on endogenous levels of
 NGFI-A mRNA, growth-quiescent SMCs were exposed to ED5 prior to
 stimulation with serum. Northern blot and densitometric analysis revealed
 30 that ED5 (0.1 μ M) inhibited serum-inducible steady-state NGFI-A mRNA
 levels by 55% (data not shown), whereas ED5SCR had no effect (data not
 shown). The capacity of ED5 to inhibit NGFI-A synthesis at the level of
 protein was assessed by Western blot analysis. Serum-induction of NGFI-A
 protein was suppressed by ED5. In contrast, neither ED5SCR nor EDC, a
 35 DNAzyme bearing an identical catalytic domain as ED5 and ED5SCR but
 flanked by nonsense arms had any influence on the induction of NGFI-A

(Figure 7). ED5 failed to affect levels of the constitutively expressed, structurally-related zinc-finger protein, Sp1 (Figure 7). It was also unable to block serum-induction of the immediate-early gene product, c-Fos (Figure 7) whose induction, like NGFI-A, is dependent upon serum response elements in its promoter and phosphorylation mediated by extracellular-signal regulated kinase (Treisman, 1990, 1994 and 1995; Gashler & Sukhatme, 1995). These findings, taken together, demonstrate the capacity of ED5 to inhibit production of NGFI-A mRNA and protein in a gene-specific and sequence-specific manner, consistent with the lack of significant homology between its target site in NGFI-A mRNA and other mRNA (Table 2).

The effect of ED5 on SMC replication was next determined. Growth-quiescent SMCs were incubated with DNase prior to exposure to serum and the assessment of cell numbers after 3 days. ED5 (0.1 μ M) inhibited SMC proliferation stimulated by serum by 70% (Figure 8a). In contrast, ED5SCR failed to influence SMC growth (Figure 8a). AS2, an antisense NGFI-A ODN able to inhibit SMC growth at 1 μ M failed to inhibit proliferation at the lower concentration (Figure 8a). Additional experiments revealed that ED5 also blocked serum-inducible 3 H-thymidine incorporation into DNA (data not shown). ED5 inhibition was not a consequence of cell death since no change in morphology was observed, and the proportion of cells incorporating Trypan Blue in the presence of serum was not influenced by either DNase (Figure 8b).

Cultured SMCs derived from the aortae of 2 week-old rats (WKY12-22) are morphologically and phenotypically similar to SMCs derived from the neointima of balloon-injured rat arteries (Seifert et al, 1984; Majesky et al, 1992). The epitheloid appearance of both WKY12-22 cells and neointimal cells contrasts with the elongated, bipolar nature of SMCs derived from normal quiescent media (Majesky et al, 1988). WKY12-22 cells grow more rapidly than medial SMCs and overexpress a large number of growth-regulatory molecules (Lemire et al, 1994), such as NGFI-A (Rafty & Khachigian, 1998), consistent with a "synthetic" phenotype (Majesky et al, 1992; Campbell & Campbell, 1985). ED5 attenuated serum-inducible WKY12-22 proliferation by approximately 75% (Figure 8c). ED5SCR had no inhibitory effect; surprisingly, it appeared to stimulate growth (Figure 8c). Trypan Blue exclusion revealed that DNase inhibition was not a consequence of cytotoxicity (data not shown).

To ensure that differences in the biological effects of ED5 and ED5SCR were not the consequence of dissimilar intracellular localization, both DNazymes were 5'-end labeled with fluorescein isothiocyanate (FITC) and incubated with SMCs. Fluorescence microscopy revealed that both FITC-ED5 and FITC-ED5SCR localized mainly within the nuclei. Punctate fluorescence in this cellular compartment was independent of DNzyme sequence. Fluorescence was also observed in the cytoplasm, albeit with less intensity. Cultures not exposed to DNzyme showed no evidence of autofluorescence.

Both molecules were 5'-end labeled with $\gamma^{32}\text{P}$ -dATP and incubated in culture medium to ascertain whether cellular responsiveness to ED5 and ED5SCR was a consequence of differences in DNzyme stability. Both ^{32}P -ED5 and ^{32}P -ED5SCR remained intact even after 48 h (data not shown). In contrast to ^{32}P -ED5 bearing the 3' inverted T, degradation of ^{32}P -ED5 bearing its 3' T in the correct orientation was observed as early as 1 h. Exposure to serum-free medium did not result in degradation of the molecule even after 48 h (data not shown). These findings indicate that inverse orientation of the 3' base in the DNzyme protects the molecule from nucleolytic cleavage by components in serum.

Physical trauma imparted to SMCs in culture results in outward migration from the wound edge and proliferation in the denuded zone. We determined whether ED5 could modulate this response to injury by exposing growth-quiescent SMCs to either DNzyme and Mitomycin C, an inhibitor of proliferation (Pitsch et al, 1996; Horodyski & Powell, 1996) prior to scraping. Cultures in which DNzyme was absent repopulated the entire denuded zone within 3 days. ED5 inhibited this reparative response to injury and prevented additional growth in this area even after 6 days (data not shown). That ED5SCR had no effect in this system further demonstrates sequence-specific inhibition by ED5.

The effect of ED5 on neointima formation was investigated in a rat model. Complete ligation of the right common carotid artery proximal to the bifurcation results in migration of SMCs from the media to the intima where proliferation eventually leads to the formation of a neointima (Kumar & Lindner, 1997; Bhawan et al, 1977; Buck, 1961). Intimal thickening 18 days after ligation was inhibited 50% by ED5 (Figure 9). In contrast, neither its scrambled counterpart (Figure 9) nor the vehicle control (Figure 9) had any

effect on neointima formation. These findings demonstrate the capacity of ED5 to suppress SMC accumulation in the vascular lumen in a specific manner, and argue against inhibition as a mere consequence of a "mass effect" (Kitze et al, 1998; Tharlow et al, 1996). Sequence specific inhibition of inducible NGFI-A protein expression and intimal thickening by ED5 was also observed in the rat carotid balloon injury model (Santiago et al., 1999a).

Further experiments revealed the capacity of hED5 to cleave (human) EGR-1 RNA. hED5 cleaved its substrate in a dose-dependent manner over a wide range of stoichiometric ratios. hED5 also cleaved in a time-dependent manner, whereas hED5SCR, its scrambled counterpart, had no such catalytic property (data not shown).

The specific, growth-inhibitory properties of antisense EGR-1 strategies reported herein suggest that EGR-1 inhibitors may be useful as therapeutic tools in the treatment of vascular disorders involving inappropriate SMC growth, endothelial growth and tumour growth.

EXAMPLE 3

Use of DNAzymes to inhibit growth of malignant cells

Materials and Methods

HepG2 cells were routinely grown in DMEM, pH 7.4, containing 10 % fetal calf serum supplemented with antibiotics. The cells were trypsinized, resuspended in growth medium (to 10,000 cells/200 μ l) and 200 μ l transferred into sterile 96 well titre plates. Two days subsequently, 180 μ l of the culture supernatant was removed, the cells were washed with PBS, pH 7.4, and refed with 180 μ l of serum free media. After 6 h, the first transfection of DNAzyme (2 μ g/200 μ l wall, 0.75 μ M final) was performed in tubes containing serum free media using FuGENE6 at a ratio of 1:3 (μ g: μ l). After 15 min incubation at room temperature, 180 μ l of the culture supernatant was replaced with 180 μ l of the transfection mix. After 24 h, 180 μ l of the supernatant was replaced with 180 μ l of new transfection mix, but this time in 5% FBS media. After 3 days, the cells were washed in PBS, pH 7.4, and resuspended by trypsinization in 100 μ l trypsin-EDTA. The cells were shaken for approximately 5 min to ensure the cells were in suspension. The entire suspension was placed into 10 ml of Isoton II. That all the cells were transferred was ensured by pipetting Isoton II solution from tubes back into wells several times. Using Isoton II only, background cell

number was determined. Each sample was counted three times and used to calculate mean counts and standard errors of each mean.

Results and Discussion

5 Our results indicate that serum stimulated HepG2 cell proliferation after 3 days (Figure 10). Proliferation was almost completely suppressed by 0.75 μ M of DzA (5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3), catalytic moiety in capitals), a DNAzyme targeting human EGR-1 mRNA (arms hybridize to nts 189-207) (Figure 10). In contrast, HepG2 cell growth was not inhibited by
10 ED5SCR (Figure 10). Western blot analysis revealed that DzA strongly inhibited EGR-1 expression in HepG2 cells, whereas a size matched DNAzyme with different sequence (5'-tcagctgcaGGCTAGCTACAACGActcggcctt) (SEQ ID NO:24) had no effect (data not shown). These data indicate that inducible proliferation of this model human malignant cell line can be blocked by the
15 EGR-1 DNAzyme. These findings suggest that EGR inhibitors may be clinically useful in therapeutic strategies targeting human cancer.

 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention
20 as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive. Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which the invention pertains.

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Claims:

1. A method for the treatment of a tumour, the method comprising administering to a subject in need thereof an agent which inhibits induction of an EGR, an agent which decreases expression of an EGR or an agent which decreases the nuclear accumulation or activity of an EGR.
2. A method as claimed in claim 1 in which the agent inhibits angiogenesis.
3. A method as claimed in claim 1 or claim 2 in which the agent directly inhibits proliferation of the tumour cells.
4. A method as claimed in any one of claims 1 to 3 in which the tumour is a solid tumour.
5. A method as claimed in any one of claims 1 to 4 in which the EGR is EGR-1.
6. A method as claimed in any one of claims 1 to 5 in which the expression of EGR is decreased.
7. A method as claimed in claim 6 in which the expression of EGR is decreased by the use of an EGR antisense oligonucleotide.
8. A method as claimed in claim 7 in which the antisense oligonucleotide has a sequence selected from the group consisting of
 - (i) ACA CTT TTG TCT GCT (SEQ ID NO:4), and
 - (ii) CTT GGC CGC TGC CAT (SEQ ID NO:2).
9. A method as claimed in claim 6 in which the expression of EGR is decreased by the cleavage of EGR mRNA by a sequence-specific ribozyme.
10. A method as claimed in claim 6 in which the expression of EGR is decreased by the use of a ssDNA targeted against EGR dsDNA the ssDNA molecule being selected so as to form a triple helix with the dsDNA.
11. A method as claimed claim 6 in which the expression of EGR is decreased by inhibiting transcription of the EGR gene using a nucleic acid transcriptional decoy.
12. A method as claimed in claim 6 in which the expression of EGR is decreased by the expression of antisense EGR mRNA .
13. A method as claimed in claim 6 in which the expression of EGR is decreased by cleavage of EGR mRNA by a sequence specific DNAzyme.
14. A method as claimed in claim 13 in which the DNAzyme comprises
 - (i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

- (ii) a first binding domain contiguous with the 5' end of the catalytic domain; and
- (iii) a second binding domain contiguous with the 3' end of the catalytic domain,

5 wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:15, such that the DNAzyme cleaves the EGR mRNA.

15 15. A method as claimed in claim 13 or claim 14 in which the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA.

16. A method as claimed in any one of claims 13 to 15 in which the cleavage site is selected from the group consisting of

- (i) the GU site corresponding to nucleotides 198-199;
- (ii) the GU site corresponding to nucleotides 200-201;
- 15 (iii) the GU site corresponding to nucleotides 264-265;
- (iv) the AU site corresponding to nucleotides 271-272;
- (v) the AU site corresponding to nucleotides 301-302;
- (vi) the GU site corresponding to nucleotides 303-304; and
- (vii) the AU site corresponding to nucleotides 316-317.

20 17. A method as claimed in claim 16 in which the cleavage site is the GU site corresponding to nucleotides 198-199, the AU site corresponding to nucleotides 271-272 or the AU site corresponding to nucleotides 301-302.

18. A method as claimed in claim 16 in which the DNAzyme has a sequence selected from the group consisting of:

- 25 (i) 5'-caggggacaGGCTAGCTACAACGAacgttgcg (SEQ ID NO:3);
- (ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO:6);
- (iii) 5'-catcctggaGGCTAGCTACAACGAagagcaggct (SEQ ID NO:7);
- (iv) 5'-ccgcgccaGGCTAGCTACAACGAacctggacga (SEQ ID NO:8);
- (v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO:9);
- 30 (vi) 5'-gcggggacaGGCTAGCTACAACGAacagctgcat (SEQ ID NO:10);
- (vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO:11); and
- (viii) 5'-ggtcagagaGGCTAGCTACAACGAactgcagcgg (SEQ ID NO:12).

19. A method as claimed in claim 18 in which the DNAzyme has the sequence: 5'-caggggacaGGCTAGCTACAACGAacgttgcg (SEQ ID NO:3) or 5'-gcggggacaGGCTAGCTACAACGAacagctgcat (SEQ ID NO:10).

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20. A method as claimed in claim 18 in which the DNAzyme has the sequence: 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8) or 5'-ccgctgcccaGGCTAGCTACAACGAcccgacgt (SEQ ID NO:9).
21. A method as claimed in any one of claims 13 to 19, wherein the 3'-end
5 nucleotide residue of the DNAzyme is inverted in the binding domain contiguous with the 3' end of the catalytic domain.
22. A method as claimed in any one of claims 1 to 21 which further comprises administering one or more additional anti-cancer agents.
23. A method for inhibiting the growth or proliferation of a tumour cell, the
10 method comprising contacting a tumour cell with an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.
24. A tumour cell which has been transformed by introducing into the cell a
15 nucleic acid molecule, the nucleic acid molecule comprising or encoding (i) an agent which inhibits induction of EGR, (ii) an agent which decreases expression of EGR, or (iii) an agent which decreases the nuclear accumulation or activity of EGR.
25. A method of screening for an agent which inhibits angiogenesis, the
20 method comprising testing a putative agent for the ability to inhibit induction of EGR, decrease expression of EGR or decrease the nuclear accumulation or activity of EGR.

1/14

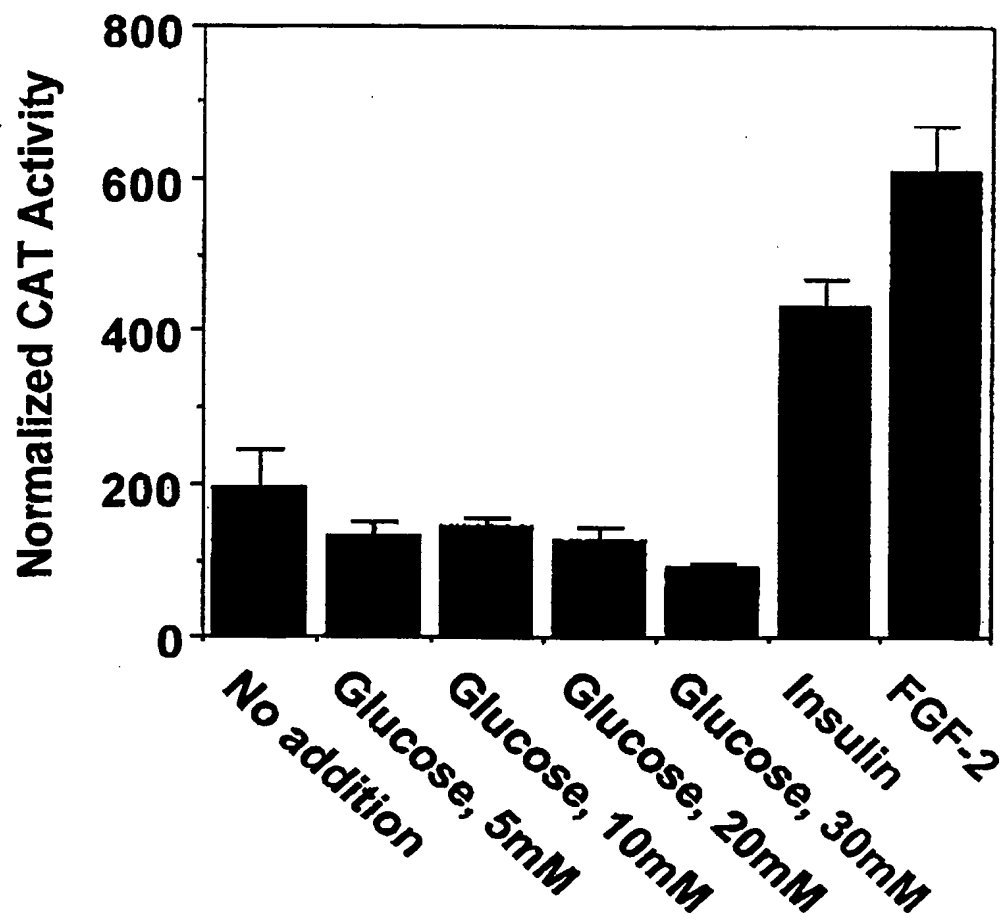


Figure 1

2/14

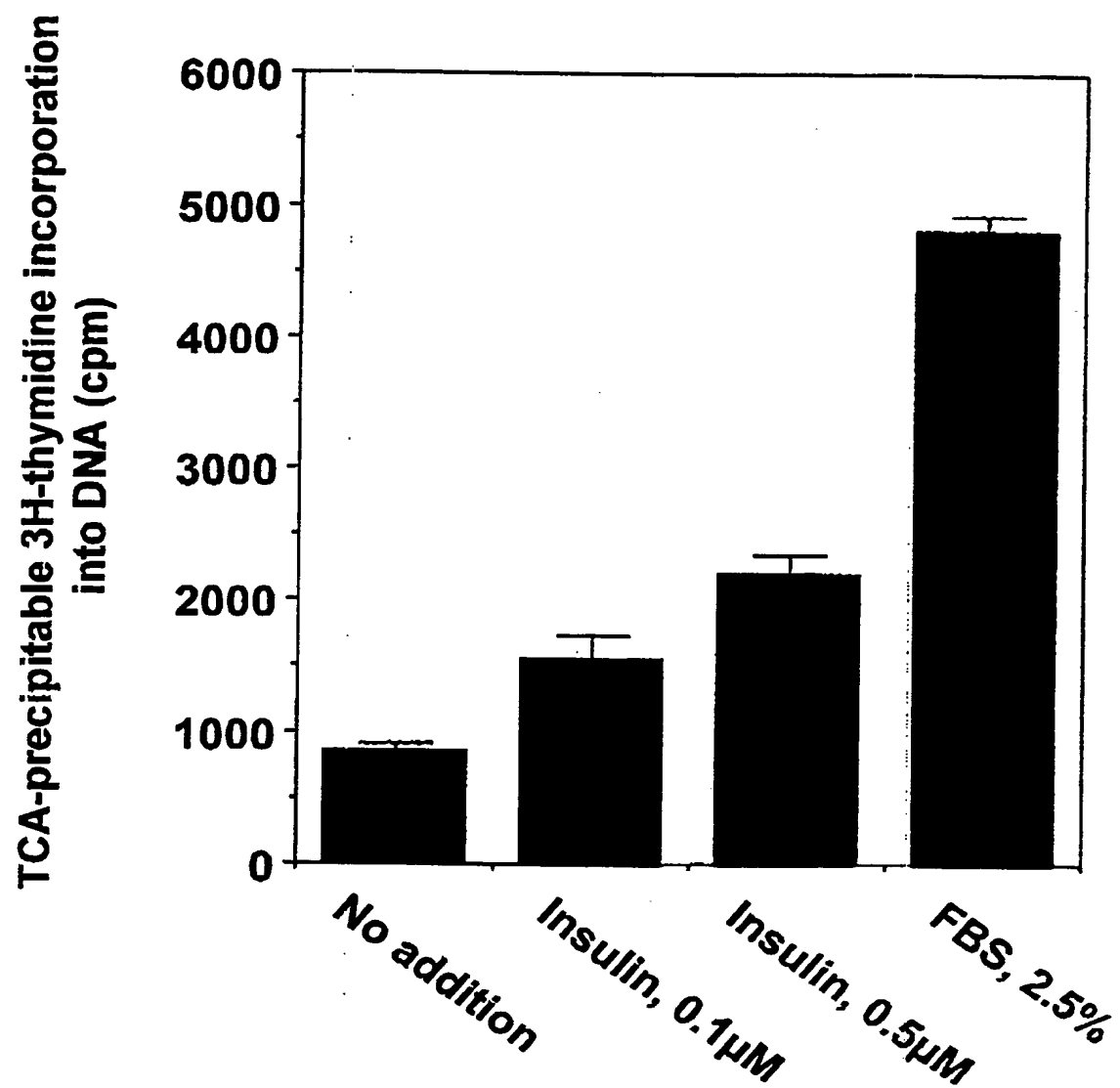


Figure 2A

3/14

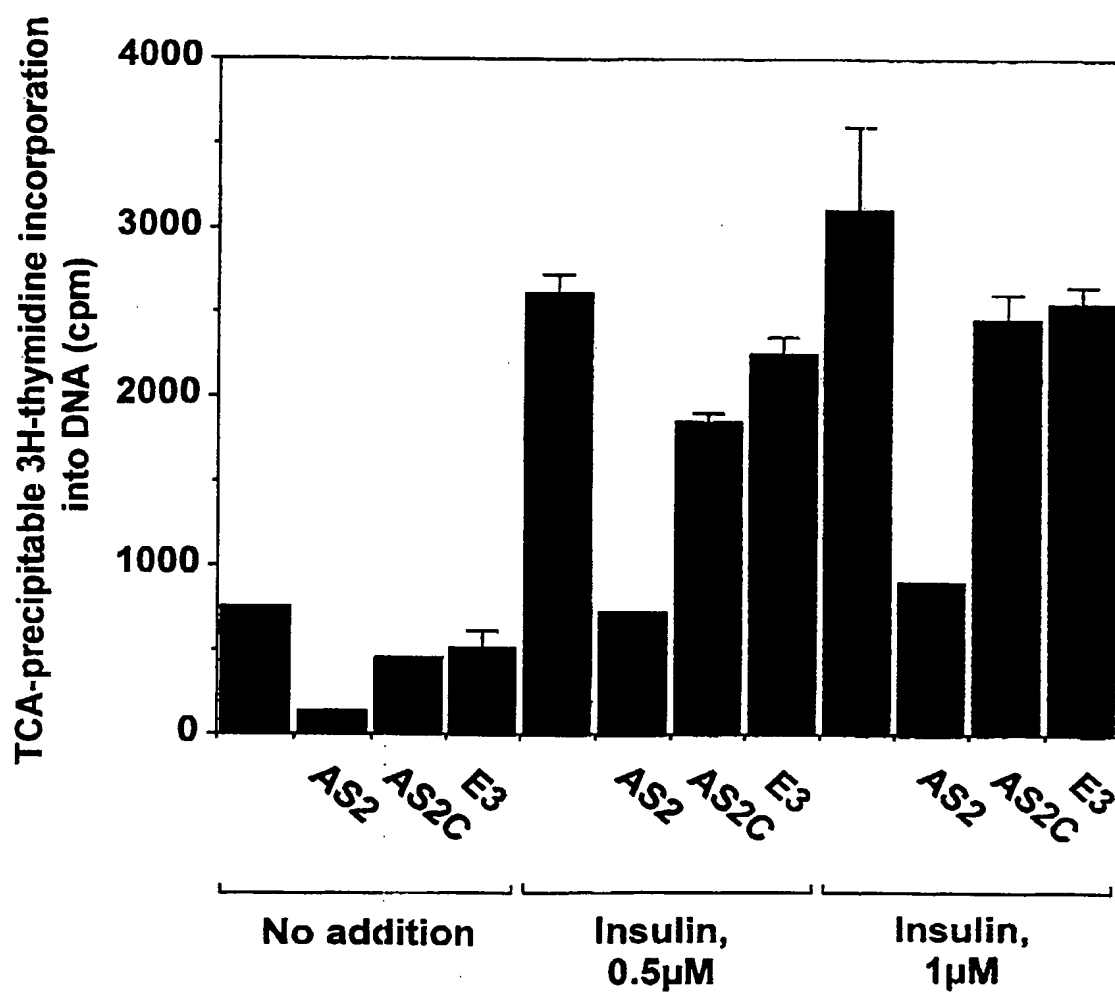


Figure 2B

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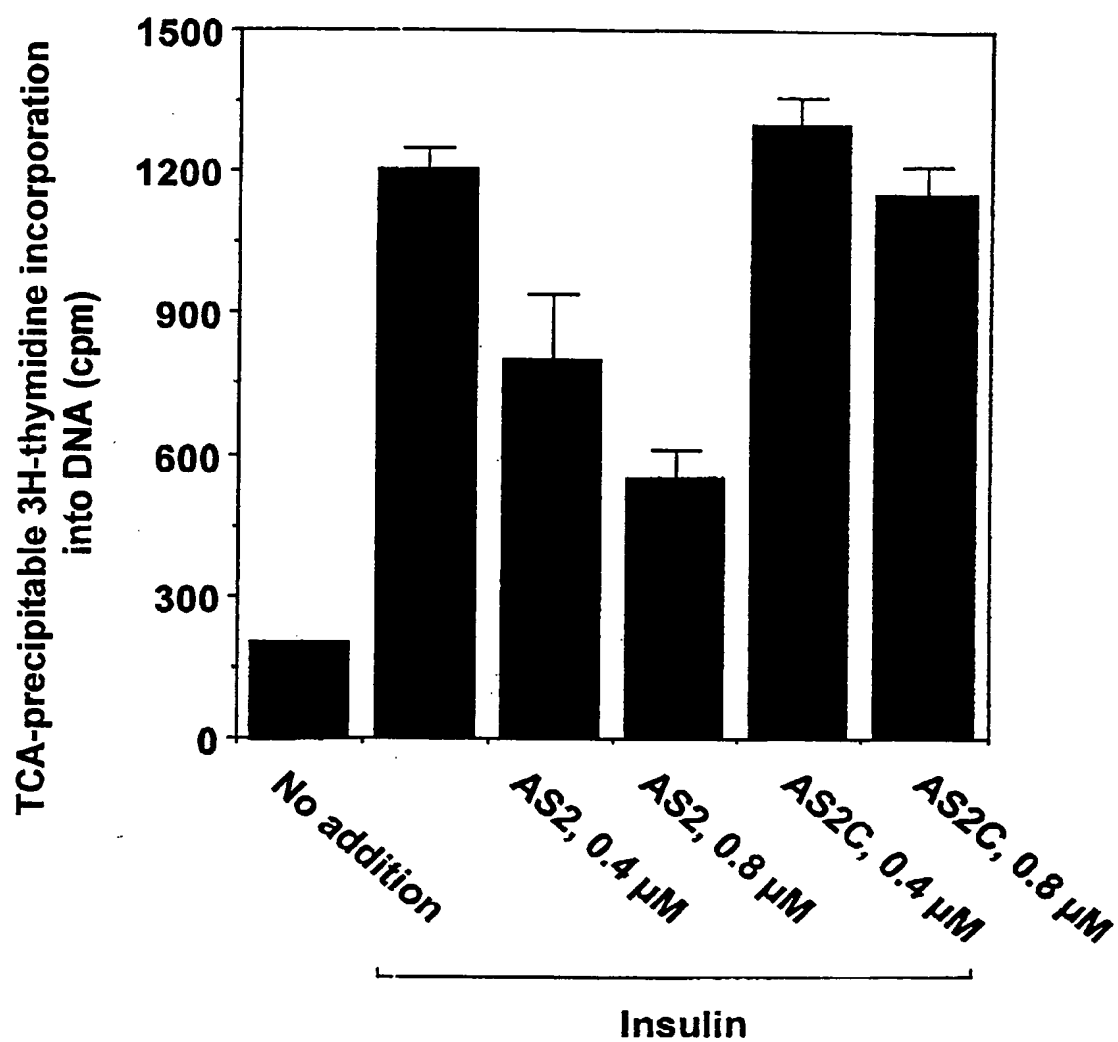


Figure 2C

5/14

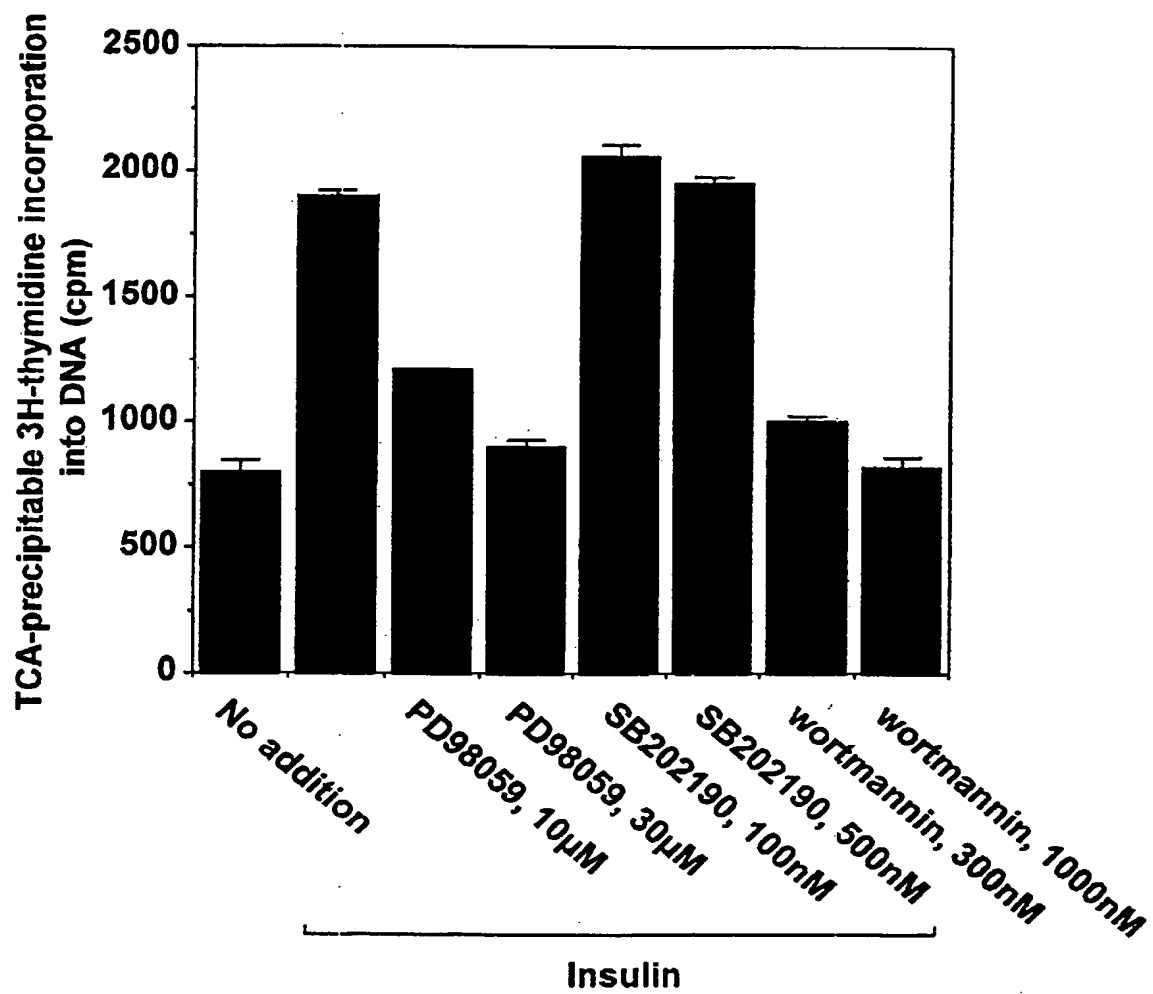


Figure 3

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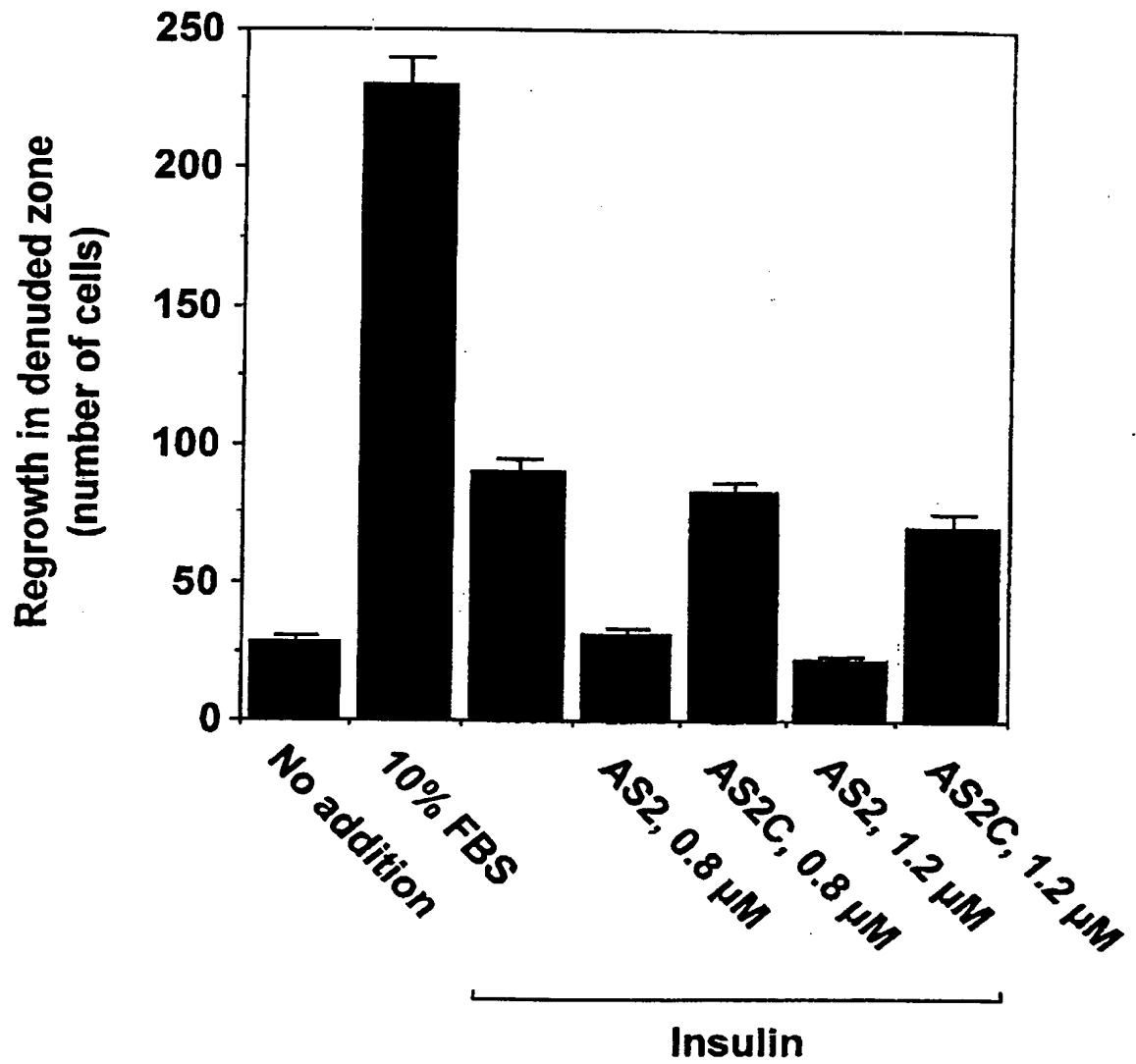


Figure 4

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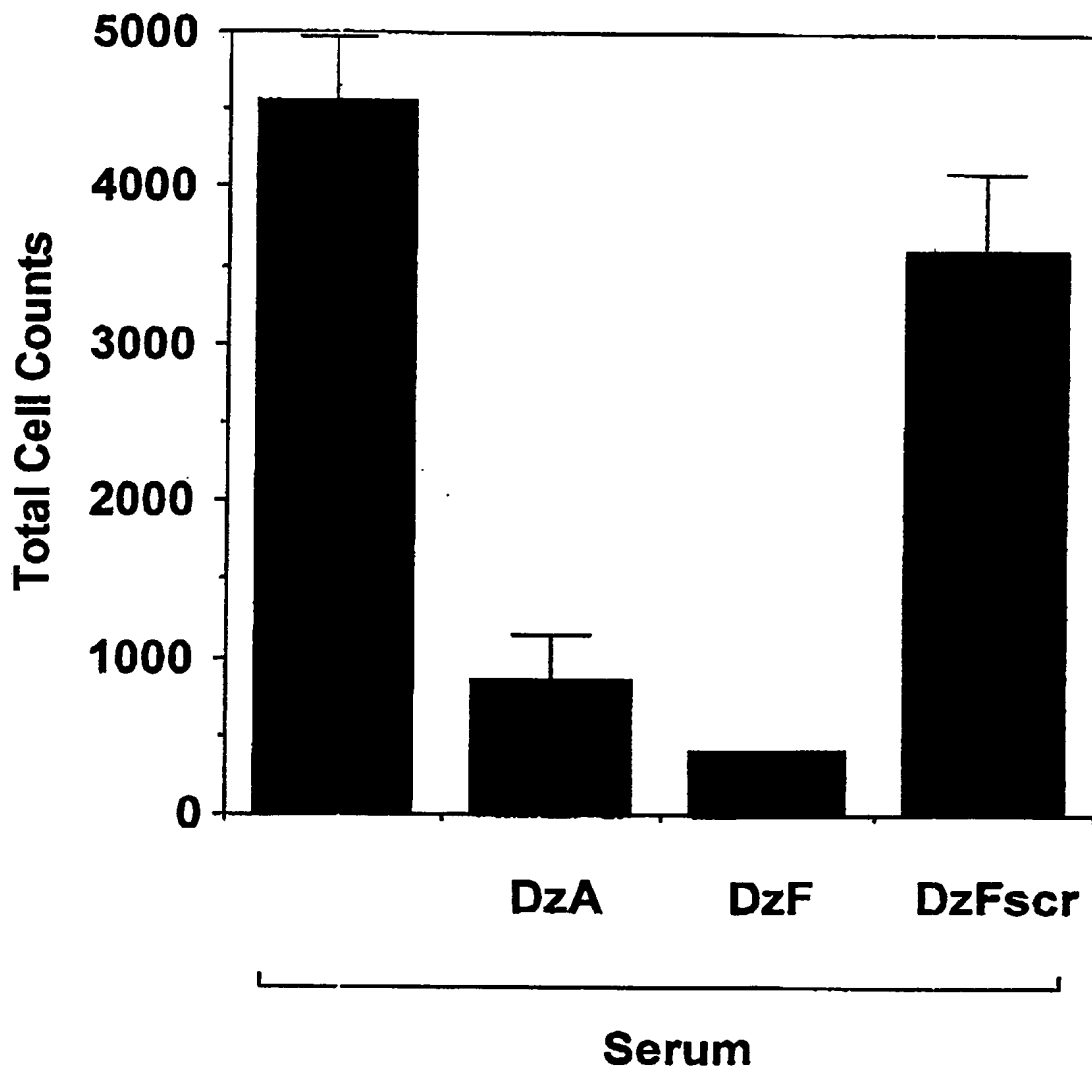
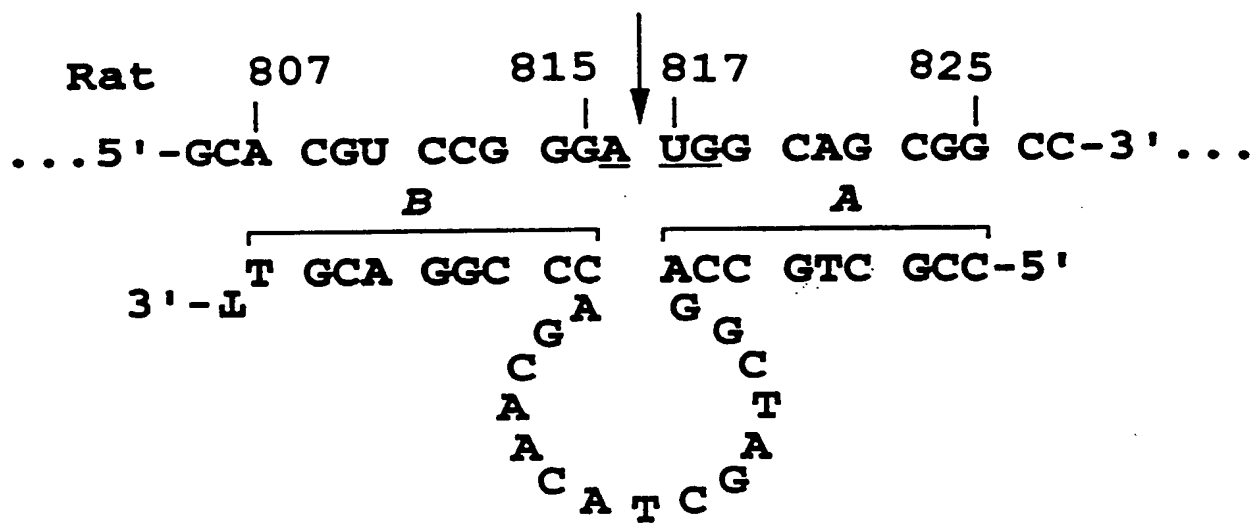
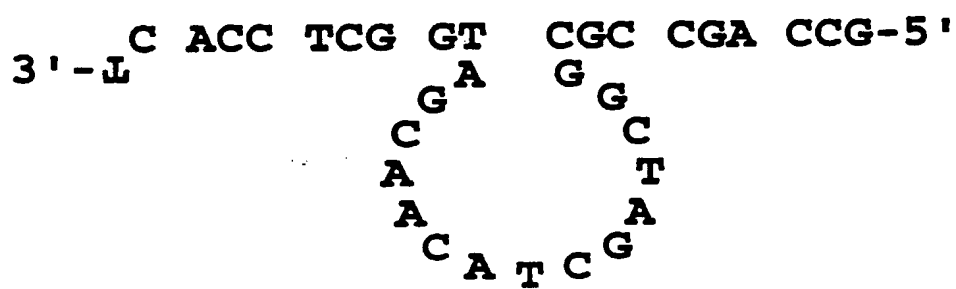


Figure 5

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ED5



ED5SCR

Figure 6

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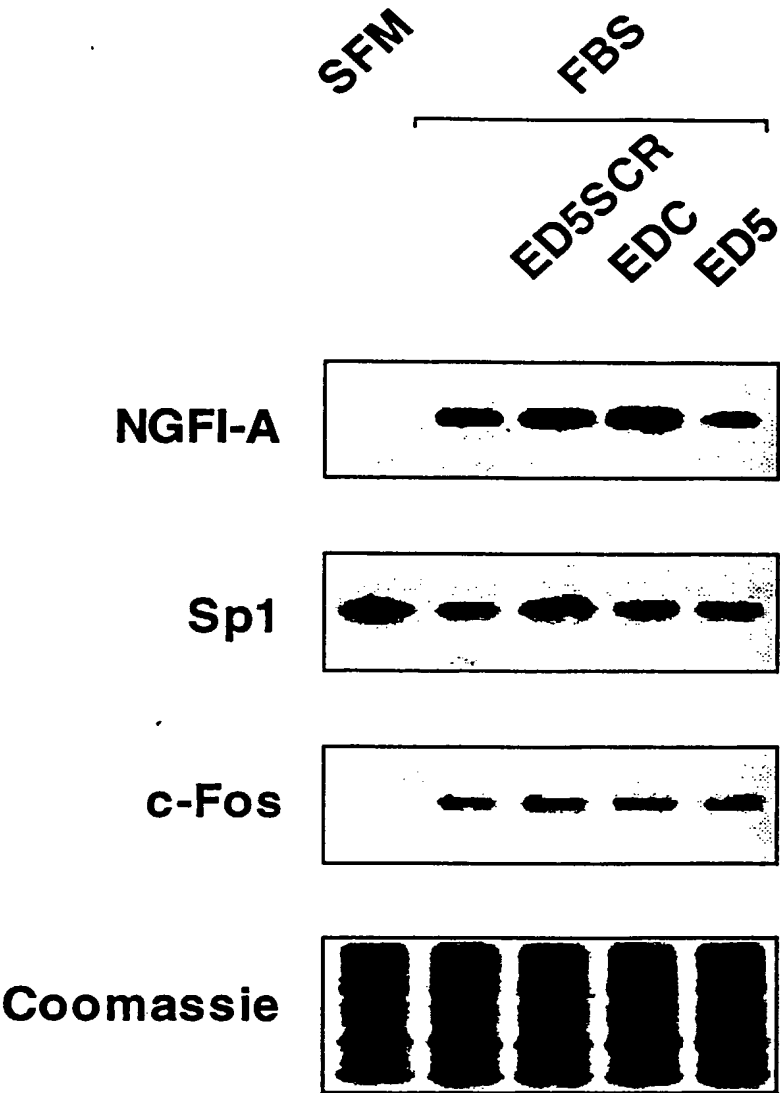


Figure 7

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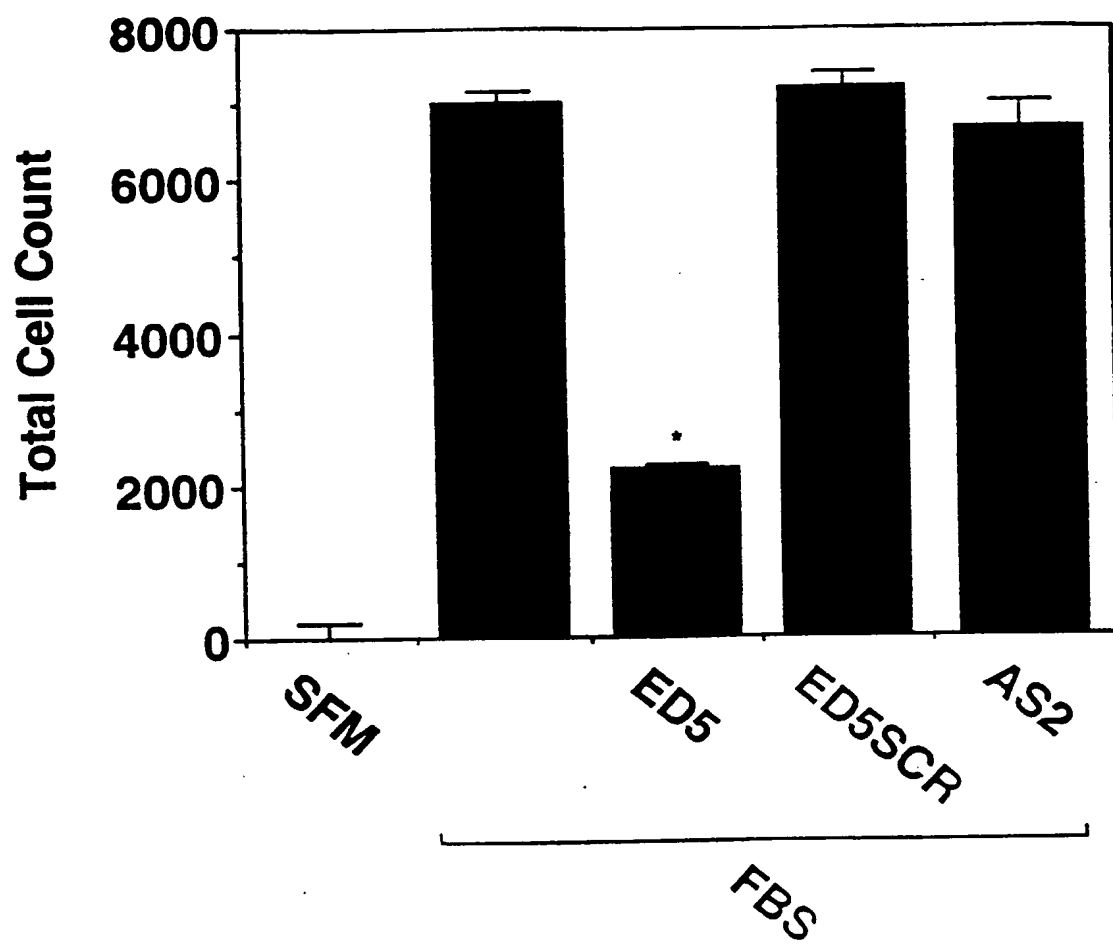


Figure 8A

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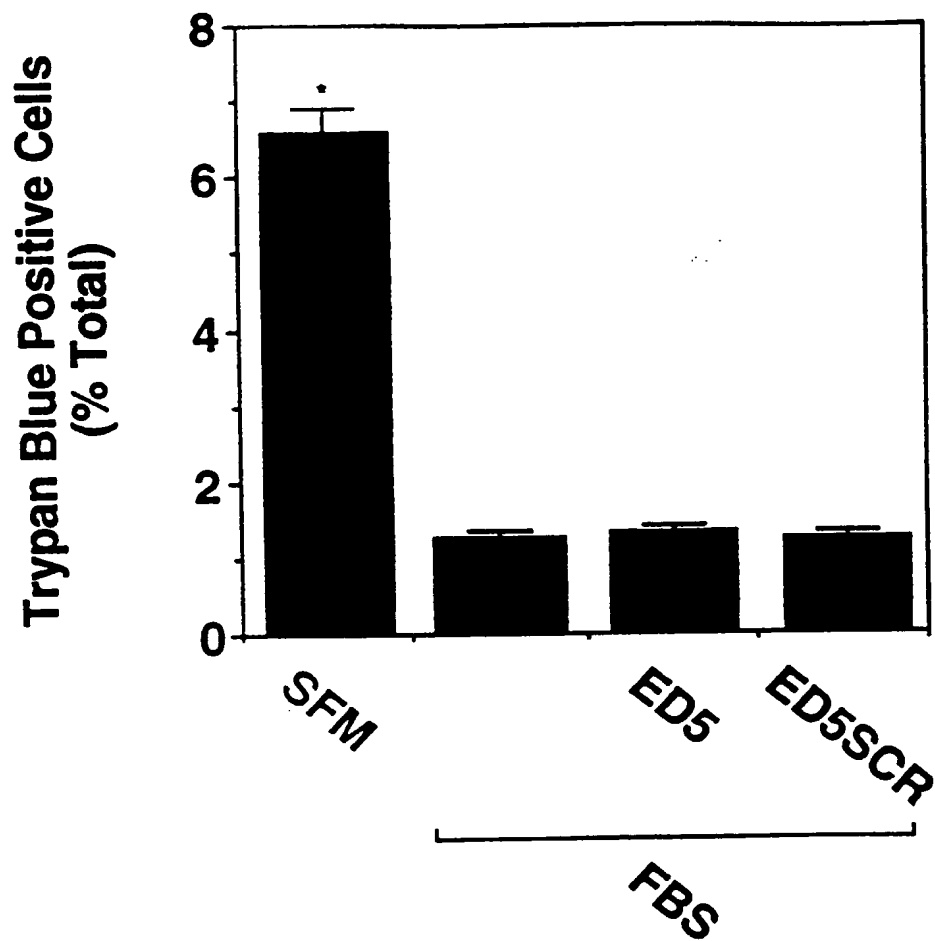


Figure 8B

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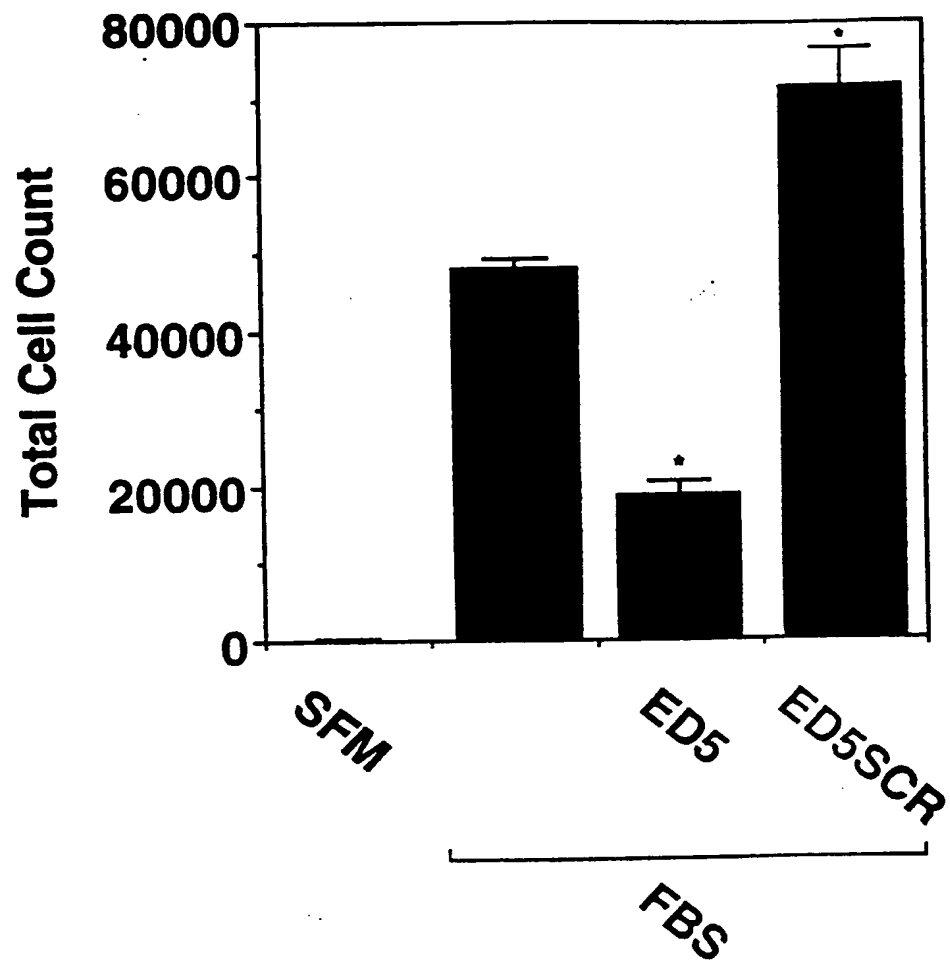


Figure 8C

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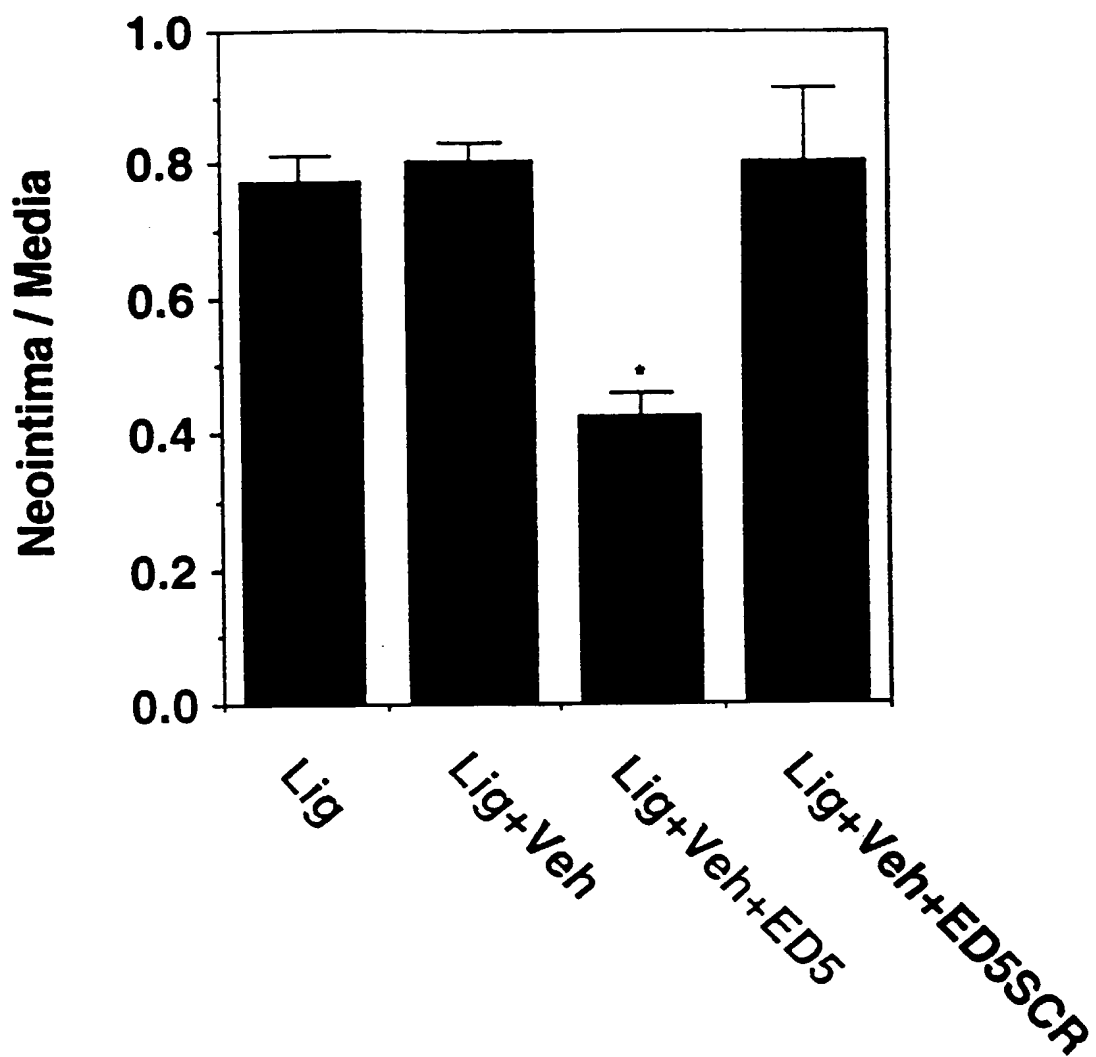


Figure 9

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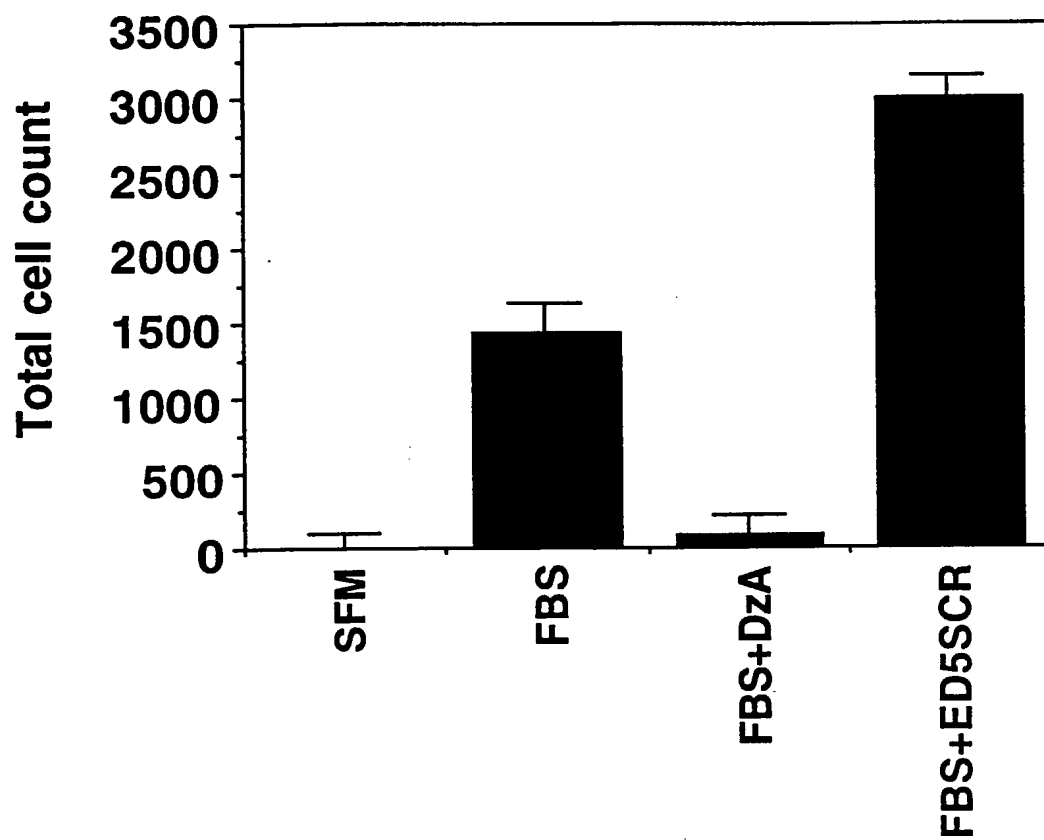


Figure 10

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3/9

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4/9

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7/9

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01315**A. CLASSIFICATION OF SUBJECT MATTER**Int. Cl. ⁷: A61K 48/00, A61P 35/00, C12N15/11, C07H 21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

DERWENT DATABASES: WPAT; CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

MEDLINE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT DATABASES: WPAT; CHEMICAL ABSTRACTS; MEDLINE - Keywords used (see Supplemental Box)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	AU, B, 20865/97 (707943) (UNISEARCH LIMITED) opi. 22 September 1997, epd. 7 March 1996 (pages 8-17 & claims)	1-13, 22-25 14-21
X Y	J. of Biological Chemistry, 268(26), 15 September 1993, 'NGFI-A Gene Expression Is Necessary for T Lymphocyte Proliferation', A. Perez-Castillo et.al., pages 19445-19450 (entire document)	1-7, 9-13, 22-25 8, 14-21
X Y	Molecular and Cellular Biology, 15(2), February 1995, 'The Zinc Finger Transcription Factor EGR-1 Impedes Interleukin-I-Inducible Tumor Growth Arrest', S.F. Sells et.al., pages 682-692 (entire document)	1-7, 9-13, 22-25 8, 14-21

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
20 December 2000Date of mailing of the international search report
28 DEC 2000Name and mailing address of the ISA/AU
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/01315

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. of Biological Chemistry, 272(44), 31 October 1997, 'Role of <i>EGR</i> -1 Expression in B Cell Receptor-induced Apoptosis in an Immature B Cell Lymphoma', S. Muthukumar et.al., pages 27987-27993 (entire document) ✓	1-7, 9-13, 22-25
Y		8, 14-21
X	Cardiovascular Pathobiology, 2(3/4), 1998, 'Prostaglandin F _{2α} -induced Growth of Neonatal Rat Cardiomyocytes is Inhibited by Antisense <i>Egr</i> -1 Oligodeoxynucleotides', J.W. Adams and S. A. Henderson, pages 167-180 (entire document) ✓	1, 2, 5-7, 9-13, 25
Y		8, 14-21
X	Cancer Research, 58, 1 June 1998, 'Expression of Early Growth Response Genes in Human Prostate Cancer', M.A. Eid et. al., pages 2461-2468 (entire document) ✓	1-7, 9-13, 22-25
X	Oncogene, 17(19), 1998, 'Wild-type <i>egr1</i> /Krox24 promotes and dominant-negative mutants inhibit, pluripotent differentiation of p19 embryonal carcinoma cells', J. Lanoix et.al., pages 2495-2504 (entire document) ✓	1-6, 22-25
X	American Journal of Pathology, 155(3), September 1999, 'Vascular Smooth Muscle Cell Proliferation and Regrowth after Mechanical Injury <i>in vitro</i> Are <i>Egr</i> -1/NGFI-A-Dependent', F.S. Santiago et.al., pages 697-905 (entire document) ✓	1, 2, 5-7, 9-13, 25
Y		8, 14-21
Y	US 5 807 718 (JOYCE et.al.) publ. 15 September 1998, epd. 7 June 1995 (entire document) ✓	8, 14-21
Y	Proc. Natl. Acad. Sci. USA, 94, April 1997, 'A general purpose RNA-Cleaving DNA enzyme', S.W. Santoro and G.F. Joyce, pages 4262-4266 (entire document) ✓	8, 14-21
P, X	AU, A, 44487/00 (MERCOLA & ADAMSON) opi. 25 August 2000, epd. 5 February 1999 (entire document) ✓	1-25
P, X	AU, A, 21444/00 (ISIS PHARMACEUTICALS INC.) opi. 26 June 2000, epd. 4 December 1998 (entire document) ✓	1-25
P, X	Nature Medicine, 5(11), November 1999, 'New DNA enzyme targeting <i>Egr</i> -1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury', F.S. Santiago et.al., pages 1264-1269 (entire document) ✓	1, 2, 5-7, 9-13, 25
P, Y		8, 14-21
A	Progress in Nucleic Acid Research & Molecular Biology, 50, 1995, 'Early-Growth Response Protein 1 (<i>Egr</i> -1: Prototype of a Zinc-finger Family of Transcription Factors', A. Gashler and V. P. Sukhatme, pages 191-224 (entire document) ✓	1-25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/01315

Supplemental Box

(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: B

Keywords used

WPAT; Chemical Abstracts - (EGR()1 or NGFI()A or ZIF368 or KROX24 or TIS8 or early growth response) AND (cancer or carcinoma or tumor or tumour or malignant or proliferation or angiogenesis or antisense or ribozyme or DNA()enzyme or DNAzyme or antibod+ or anti()bod+ or antagonist#)

Medline - (early()growth()response()factor()1 or EGR()1 or NGFI()A or ZIF368 or KROX24 or TIS8) AND (cancer or carcinoma or tumor or tumour or malignant or prolif? or angiogen? or antisense or ribozyme or DNA()enzyme or DNAzyme or antibod? or anti()bod? or decreas? or reduc? or bind?)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/01315

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
AU A	20865/97	AU B	707943	WO	97/32979		
		EP	934404	CA	2248350		
AU A	44487/00	WO	00/45771				
AU A	21444/00	WO	00/34302	US	6008048		
US	5807718	AU	45950/96	AU B	710747	BR	9510003
		CA	2205382	CN	1173207	EP	792375
		FI	972333	HU	77576	NO	972483
		WO	96/17086	US	5807718		
END OF ANNEX							